For Reference

NOT TO BE TAKEN FROM THIS ROOM

Ex libris
universitatis
albertaensis

QUAECUMQUE VERA

For Reference

NOT TO BE TAKEN FROM THIS ROOM

Digitized by the Internet Archive
in 2020 with funding from
University of Alberta Libraries

https://archive.org/details/Danziger1968

THE UNIVERSITY OF ALBERTA

# THE INTERACTION OF AN RNA BACTERIOPHAGE (R17) WITH F-PILI

by

© RICHARD E. DANZIGER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

APRIL, 1968

Thesis
1968 (F)
24 D

UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE INTERACTION OF AN RNA BACTERIOPHAGE (R17) WITH F-PILI", submitted by Richard E. Danziger in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

# ABSTRACT

This thesis presents the results of studies carried out to characterize the interaction of an RNA bacteriophage (R17) with F-pili, the proposed phage receptors on male strains of the bacterium *Escherichia coli*.

The effect of various cations on the attachment of the phage to cell-free F-pili was studied using a filtration technique. The attachment of $P^{32}$-labelled phage to pili was monitored by trapping $P^{32}$-labelled R17-pili complexes on a filter membrane, with subsequent determination of the radioactivity of the filter. It was found that phage attachment to F-pili displays a non-specific requirement for mono- or divalent cations, an ionic strength of 0.08 or greater giving rise to optimal phage-pili interaction.

Using the same filtration technique, the nature of pili elongation was examined to determine whether F-pili continuously grow out from the cell and detach, or whether they grow to a fixed length and remain associated with the bacterial cell. It was found that F-pili were extremely sensitive to shearing forces, but when culture conditions were established, in which little artificial pili breakage occurred, it was found that cell-free pili represent less than 10% of the total pili produced in a growing culture.

It was therefore concluded that there is little or no spontaneous detachment of F-pili from the bacterial cell in a normally growing culture.

It was found that cell-free F-pili, unlike cell-associated F-pili, do not cause the release of viral RNA from intact bacteriophage. The difference in the two cases may, in part, be structural, but there is apparently an energy requirement for the uncoating of the phage RNA. This was shown by the fact that, in cultures grown on a glycerol carbon source, the degree of uncoating of phage RNA could be correlated with the level of ATP in the cells. However, cultures which were grown on glucose as a carbon source were found to uncoat phage RNA efficiently, even under conditions where the concentration of ATP was decreased by a factor of 90%. This latter observation was taken to mean that an energy source other than ATP is produced in glucose-grown cells and that this energy source is capable of causing uncoating of phage RNA.

The addition of glucose or its analogue, 2-deoxy-D-glucose, to glucose-starved cells also caused inhibition of the uncoating reaction, and this is discussed in the light of a possible competition between phage and sugar molecules for a membrane-associated high energy compound.

Uncoating of the phage RNA in normal infections is usually followed by a penetration of the RNA into the bacterial cell, with the bacteriophage coat remaining outside the cell. Preliminary studies have revealed that this coat protein remains essentially intact (as empty phage capsids) after the loss of the nucleic acid moiety, and that these ghosts probably do not remain attached to F-pili.

# ACKNOWLEDGEMENTS

The author wishes to express his most sincere appreciation to Dr. W. Paranchych for his patient and critical guidance throughout the course of these studies.

The stimulating and enlightening discussions with Dr. S. Igarashi are gratefully acknowledged.

The author also wishes to express a debt of gratitude to Dr. T. Yamamoto of the Department of Microbiology, who gave freely of his time to perform the excellent electron microscopy.

Thanks are also due to Miss Joan Naubert and Mr. Ronald Tsujikawa for assistance at various stages of this work, and to Mr. Roger Bradley for excellent technical and drafting assistance.

Special appreciation is extended to his wife, Berna, for typing of the final manuscript.

## TABLE OF CONTENTS

Page

Abstract . . . . . . . . . . . . . . . . iii

Acknowledgements . . . . . . . . . . . . vi

Table of Contents . . . . . . . . . . . . vii

List of Tables . . . . . . . . . . . . . xii

List of Plates . . . . . . . . . . . . . xii

List of Figures . . . . . . . . . . . . . xiii

List of Abbreviations . . . . . . . . . . xvii

I. INTRODUCTION . . . . . . . . . . . . . 1

II. PREPARATION AND PURIFICATION OF BACTERIOPHAGE R17 . . . . . . . . . . . . . . . . 19

Minimal medium for the growth of bacteria . 20

Bacterial strains . . . . . . . . . 20

Enumeration of bacterial cells . . . . . . 21

(a) Plate counts . . . . . . . . . 21

(b) Spectrophotometric method . . . . 21

Enumeration of bacteriophage . . . . . 21

(a) Plaque assay . . . . . . . . . 21

(b) Spectrophotometric determination . . 22

Preparation and purification of the bacteriophage R17 . . . . . . . . . . . . 22

Preparation of radioactive R17 . . . . . 24

Properties of the purified bacteriophage . 25

Page

III. IONIC REQUIREMENTS FOR THE ATTACHMENT OF PHAGE R17 TO CELL-FREE PREPARATIONS OF F-PILI . . 28

Introduction . . . . . . . . . . . . . 28

Methods and Materials . . . . . . . . . . 31

Preparation of cell-free F-pili . . . . 32

Filtration assay . . . . . . . . . . 33

Results . . . . . . . . . . . . . 34

(1) The effect of filter composition on the retention of free phage under various salt concentrations . . . 34

(2) The effect of the salt composition of the wash solution on dissociation of preformed phage-pili complexes . 35

(3) The stability of F-pili under normal storage conditions . . . . . . 39

(4) The standard assay for the determination of the ionic requirements for the attachment of phage R17 to F-pili 40

(a) The effect of monovalent cations 43

(b) The effect of divalent cations . 44

(c) The effect of trivalent cations 48

(d) The action of mono- and divalent cations as a function of ionic strength . . . . . . . . . 49

(e) The effect of mixed cations on the adsorption of phage . . . 51

(f) The effect of sucrose on the attachment of phage to F-pili . 54

(5) The physical state of F-pili during phage adsorption . . . . . . . . 55

Page

III. IONIC REQUIREMENTS (continued)

Discussion . . . . . . . . . . . . . 60

IV. THE PHYSIOLOGICAL ASPECTS OF F-PILI GROWTH . 65

Introduction . . . . . . . . . . . . . 65

Methods and Materials . . . . . . . . . . 67

The effect of various shearing forces on the proportion of free and connected F-pili in a culture of _E. coli_ K12 . . . 69

(a) The effect of pipetting, or passing of the culture through a narrow bore needle on the state of F-pili in a bacterial culture . . . . . . 71

(b) The effect of aeration on the state of F-pili in a growing culture . . 76

An experiment to test the "continuous growth" and "limited growth" hypotheses of F-pili growth . . . . . . . . . 78

Reversibility of phage adsorption to cell-free F-pili . . . . . . . . . . 81

Discussion . . . . . . . . . . . . . 85

V. ENERGY REQUIREMENTS FOR THE UNCOATING OF BACTERIOPHAGE R17 RIBONUCLEIC ACID DURING THE EARLY STAGE OF INFECTION OF _ESCHERICHIA COLI_ K12 88

Introduction . . . . . . . . . . . . . 88

Methods and Materials . . . . . . . . . . 95

Media . . . . . . . . . . . . . . . 95

Uncoating assay . . . . . . . . . . . 96

Preparation of the firefly enzyme . . . 100

ATP assay . . . . . . . . . . . . . 101

Page

V. ENERGY REQUIREMENTS (continued)

(a) Extraction . . . . . . . . . . . 101

(b) Assay . . . . . . . . . . . . . 102

Determination of the dry weight of *E. coli* K12 . . . . . . . . . . . . . . 105

The effect of Chloramphenicol and Streptomycin on the ATP levels of *E. coli* K12 . 107

Operation of the anaerobic culture flask . 110

Results . . . . . . . . . . . . . . 113

Site of phage uncoating: the effect of blending on the ability of *E. coli* K12 to cause uncoating of phage R17 RNA . . . 113

The relationship of the F-pilus to the cell membrane . . . . . . . . . . 115

The effect of temperature on the uncoating of R17 RNA . . . . . . . . . . . . 116

The effect of anaerobiosis on uncoating of R17 RNA . . . . . . . . . . . . . 123

The effect of 2,4-dinitrophenol on the uncoating reaction . . . . . . . . . . 129

The effect of sodium arsenate on the uncoating of R17 RNA . . . . . . . . . 129

The effect of 2-deoxy-D-glucose on uncoating of phage R17 RNA by *E. coli* K12 . 135

Uncoating of phage RNA by glucose-starved *E. coli* K12 . . . . . . . . . . . 139

The effect of adenosine triphosphates on uncoating of R17 RNA . . . . . . . . 142

Discussion . . . . . . . . . . . . . 143

Page

VI. THE FATE OF THE BACTERIOPHAGE COAT PROTEIN IN THE INFECTION OF *E. COLI* K12 WITH R17 . . . 148

Introduction . . . . . . . . . . . . . . 148

Methods and Materials . . . . . . . . . . 149

Results . . . . . . . . . . . . . . . 151

The ribonuclease sensitivity of RNA phage . 151

The fate of the bacteriophage coat protein during the infection of *E. coli* K12 with R17 . . . . . . . . . . . . . . . 158

Discussion . . . . . . . . . . . . . . 165

SUMMARY . . . . . . . . . . . . . . . . 168

BIBLIOGRAPHY . . . . . . . . . . . . . . 172

## LIST OF TABLES

Table | Page
I. The effect of salt concentration on retention of $P^{32}$-labelled R17 phage by B-6 (Schleicher and Schull) and GA-6 (Gelman) filters . . . . . . . . . . . . . . 36

II. Relationship between inhibition of R17 RNA uncoating and overall levels of ATP in *E. coli* treated with metabolic inhibitors . 145

III. Distribution of radioactivity in sucrose gradients of $H^3$-$C^{14}$-labelled R17-infected cultures of *E. coli* K12 . . . . . . . 162

## LIST OF PLATES

Plate | Facing Page
I. Electron micrograph of phage R17 . . . . 4

II. The appearance of CsCl concentrated F-pili under the electron microscope . . . . . 59

III. Electron micrographs of RNase-treated phage R17 . . . . . . . . . . . . . . 155

# LIST OF FIGURES

| Figure | | Facing Page |
|---|---|---|
| 2.1 | Schlieren photographs taken during the course of sedimentation of purified phage R17 in the analytical ultracentrifuge . . | 26 |
| 3.1 | The effect of the salt composition of the wash solution on dissociation of preformed phage-pili complexes . . . . . . . . | 38 |
| 3.2 | The effect of storage in ice on the ability of F-pili to absorb phage . . . . . . | 41 |
| 3.3 | The effect of increasing concentrations of KCl on the attachment of R17 bacteriophage to F-pili . . . . . . . . . . . . | 42 |
| 3.4 | The effect of monovalent cations on the attachment of R17 bacteriophage to F-pili . | 45 |
| 3.5 | The effect of divalent cations on the attachment of R17 bacteriophage to F-pili . . . | 46 |
| 3.6 | The effect of $FeCl_3$ on the attachment of R17 bacteriophage to F-pili . . . . . . . | 47 |
| 3.7 | Adsorption of R17 to F-pili as a function of ionic strength of the incubation medium . . | 50 |
| 3.8 | The effect of sub-optimal amounts of divalent cations on the adsorption of R17 bacteriophage to F-pili in the presence of optimal levels of NaCl . . . . . . . . . . | 52 |
| 3.9 | The effect of sub-optimal amounts of mixed mono- and divalent cations on the adsorption of R17 bacteriophage to F-pili . . . . . | 53 |
| 3.10 | Cesium chloride equilibrium density gradient sedimentation of F-pili . . . . . . . | 58 |
| 3.11 | The effect of various mono- and divalent cation concentrations on the sedimentation characteristics of F-pili in sucrose gradients | 61 |

# LIST OF FIGURES (continued)

Figure — Facing Page

4.1 The effect of phage resistant (non-adsorbing) *E. coli* cells in the standard filtration assay of free F-pili . . . . . 70

4.2 Schematic diagram of the subdivision of a bacterial culture into fractions for the assay of free and connected F-pili after various shearing treatments . . . . . . 72

4.3 The effect of various shearing forces on the amount of cell-associated F-pili in a culture of *E. coli* K12 . . . . . . . . . . 74

4.4 The effect of various shearing forces on the amount of cell-free F-pili in a culture of *E. coli* K12 . . . . . . . . . . . 75

4.5 The effect of aeration on the proportion of free and connected F-pili in a growing culture of *E. coli* K12 . . . . . . . . . 77

4.6 Amounts of total and cell-free F-pili in a growing culture of *E. coli* K12 . . . . . 80

4.7 The effect of doubling phage input (at high phage/pili ratios) on the filtration assay for free F-pili . . . . . . . . . . 83

4.8 The effect of increasing phage input (at high phage/pili ratios) on the attachment of phage to free F-pili . . . . . . . . 84

5.1 Uncoating of R17 nucleic acid by cells of *E. coli* K12 . . . . . . . . . . . 90

5.2 Uncoating of bacteriophage R17 as measured by solubilization of its nucleic acid in TCA by Ribonuclease A . . . . . . . . . 98

5.3 Calibration curve for the assay of μμ mole quantities of ATP . . . . . . . . . 104

LIST OF FIGURES (continued)

| Figure | | Facing Page |
|---|---|---|
| 5.4a | The effect of time before neutralization on the extraction of ATP from *E. coli* K12 . . . | 106 |
| 5.4b | The stability of extracted ATP in the chilled perchlorate supernatant . . . . . . . . . . | 106 |
| 5.5 | Time course of the effect of Streptomycin and Chloramphenicol on ATP levels in *E. coli* K12 grown in glycerol TMM . . . . . . . . . . | 109 |
| 5.6 | Schematic diagram of the anaerobic culture flask . . . . . . . . . . . . . . . . . . | 111 |
| 5.7 | Effect of blending on the ability of cells to cause uncoating of phage R17 RNA . . . . . | 114 |
| 5.8 | Effect of temperature on uncoating of R17 RNA by *E. coli* K12 . . . . . . . . . . . . . | 118 |
| 5.9 | The effect of prechilling on the ability of *E. coli* K12 to uncoat R17 RNA . . . . . . . | 119 |
| 5.10 | The effect of anaerobiosis on ATP levels in *E. coli* K12 . . . . . . . . . . . . . . . | 126 |
| 5.11 | The effect of anaerobiosis on uncoating of R17 RNA by *E. coli* K12 . . . . . . . . . . . | 128 |
| 5.12 | The effect of sodium arsenate on the ability of *E. coli* K12 (grown in glycerol TMM) to uncoat R17 RNA . . . . . . . . . . . . | 132 |
| 5.13 | The effect of sodium arsenate on the ability of *E. coli* K12 (grown in glucose TMM) to uncoat R17 RNA . . . . . . . . . . . . | 133 |
| 5.14 | The effect of sodium arsenate on the ATP levels in *E. coli* K12 . . . . . . . . . . | 134 |
| 5.15 | The relationship between ATP levels and uncoating in arsenate-treated cultures of *E. coli* K12 . . . . . . . . . . . . . . . | 136 |

LIST OF FIGURES (continued)

| Figure | | Facing Page |
|---|---|---|
| 5.16 | The effect of 2-deoxy-D-glucose on the uncoating of R17 by *E. coli* K12 . . . . . . | 138 |
| 5.17 | The effect of glucose or 2-deoxy-D-glucose on the uncoating of R17 RNA by a glucose-starved culture of *E. coli* K12 . . . . . . . . | 140 |
| 5.18 | The level of ATP in *E. coli* K12 during carbon starvation and replenishment . . . . . . | 141 |
| 6.1 | Sucrose gradient sedimentation analysis of nucleic acid-free phage protein produced upon RNase treatment of intact phage . . . . . | 153 |
| 6.2 | Sucrose gradient profile of an R17-infected culture of *E. coli* K12 . . . . . . . . | 160 |
| 6.3 | Sucrose gradient profile of an R17-infected culture of *E. coli* K12 after blending . . . | 161 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| RNA | ribonucleic acid |
| DNA | deoxyribonucleic acid |
| RNase | Ribonuclease A (pancreatic) |
| ATP, CTP, GTP, UTP | adenosine, cytosine, guanosine, uridine 5'-triphosphate |
| ADP, CDP, GDP, UDP | adenosine, cytosine, guanosine, uridine 5'-diphosphate |
| AMP, CMP, GMP, UMP | adenosine, cytosine, guanosine, uridine 5'-monophosphate |
| TCA | trichloroacetic acid |
| PCA | perchloric acid |
| PTA | phosphotungstic acid |
| TMM | Tris-maleate minimal salts medium |
| Tris | Tris (hydroxymethyl) aminomethane |
| 2, 4-DNP | 2, 4-dinitrophenol |
| 2DG | 2-deoxy-D-glucose |
| CM | Chloramphenicol |
| Sm | Streptomycin sulfate |
| PFU | plaque forming unit |
| $\mu$ | ionic strength expressed as $\frac{1}{2}\sum c_i Z_i^2$ where $c_i$ is the concentration (molar) of the $i^{th}$ component, and $Z_i$ is its charge |
| $A_{650}$, $A_{260}$ | ultraviolet absorbancy of a solution (1 cm. light path) at 650 m$\mu$ (260 m$\mu$) |

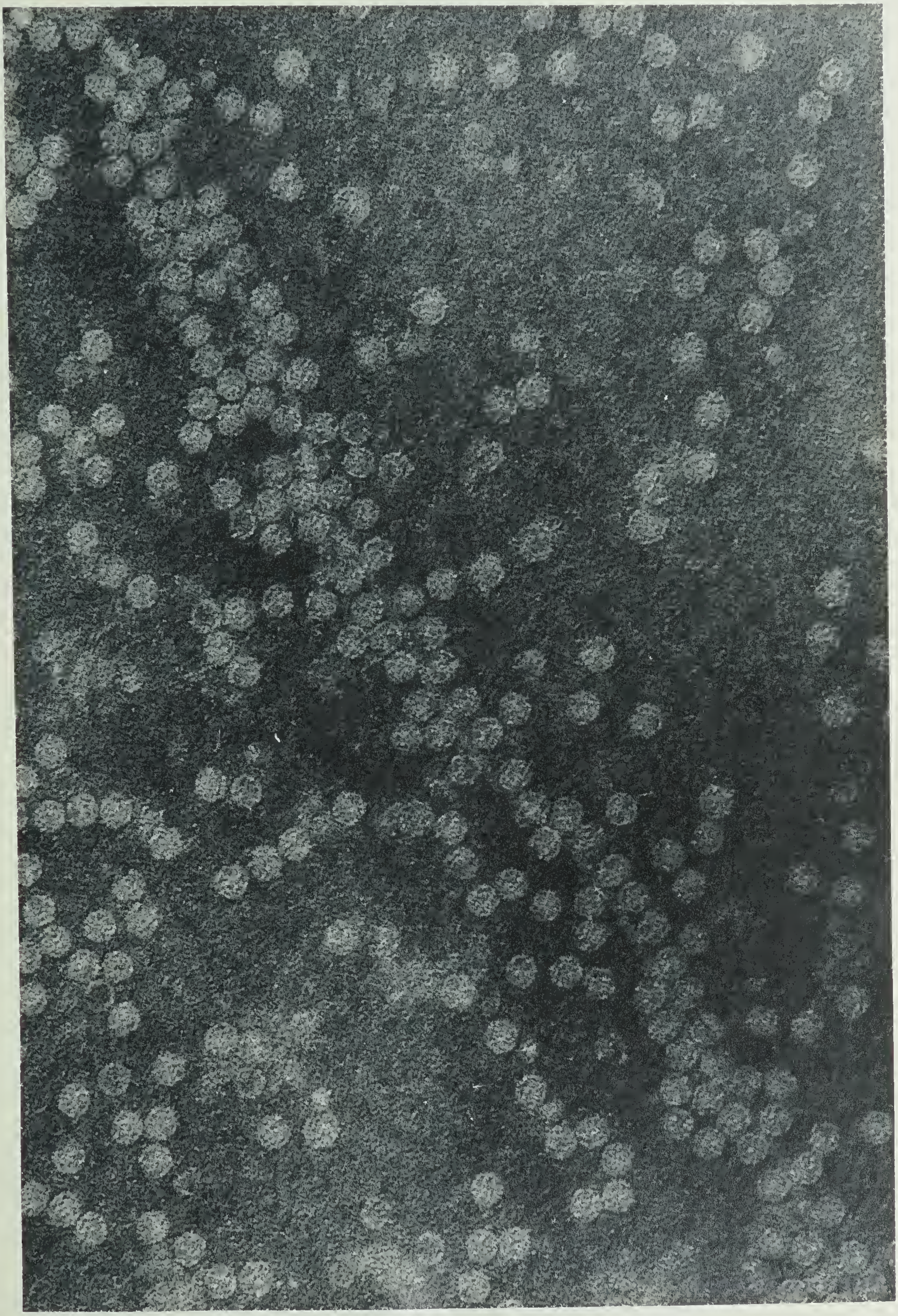
S (following a number) - sedimentation coefficient

cpm - counts per minute

rpm - revolutions per minute

nmoles - nanomoles ($10^{-9}$ moles)

All temperatures expressed as degrees centigrade except where noted otherwise.



# I. INTRODUCTION

RNA bacteriophage were found accidentally during the course of screening sewage isolates for phage that were specific for the male mating types of *Escherichia coli* (Loeb, 1961); this first RNA coliphage was designated f2. The value of such a discovery cannot be over-estimated. Viruses of all types were being used as tools of the new molecular biology since they provided discrete packets of genetic information several orders of magnitude less complex than the nucleic acid of their host organism. Ribonucleic acid as the viral genome was not unknown in the plant viruses, but the rapidity of the viral replicative events within a bacterium, whose genetics and metabolism were highly defined, was expected to lead to a relatively swift solution to the question of viral replication in general. The amount of interest raised by the isolation of f2 is well reflected by the number of workers in all parts of the world who set out to isolate a bacteriophage with similar properties and specificities: (Davis, Strauss and Sinsheimer, 1961; Paranchych and Graham, 1962; Nonoyama *et al*., 1962; Fouace and Huppert, 1962; Marvin and Hoffmann-Berling, 1963; Hofschneider, 1963; Davern, 1964; Bishop and Bradley, 1965).

A number of these phages were shown by Scott (1965) to display serological cross-reactivity when tested for

neutralization by anti-f2, anti-MS2, and anti-M12 rabbit sera. Ouchterlony double diffusion tests indicated that, although the various phage were antigenically similar, they possessed distinct antigens. Contrasted with these findings was the isolation by Watanabe et al. (1964) of a serologically distinct RNA bacteriophage, Qβ.

Physical studies by Overby et al. (1966 a & b) comparing Qβ and the antigenically dissimilar virions have pointed out differences in surface charge, base composition of the nucleic acid, and in the induced RNA replicase. Comparative studies of the mode of infection of these two types of phage may give some insight into basic essential characteristics of an RNA virus which confer upon it its infectious properties.

Although RNA phages are commonly associated with male-specific strains of E. coli, an RNA-containing Pseudomonas phage (7s) has been isolated by Feary et al. (1963). Electron micrographs of the phage show it to be morphologically similar to the RNA coliphages, however, it does not infect E. coli B, C or K12. Phage 7s has not been further characterized to any great extent, and further general discussions of RNA phage in this thesis will deal with the serologically similar RNA coliphages.

The main features distinguishable from electron micrographs of phosphotungstate stained bacteriophage are the spherical capsid and the complete absence of any tail structure (Plate I). The average diameter of the particle falls in the range 20 to 22.5 mμ. A study of the morphology of phage R17 was recently reported by Vasquez et al. (1966). Using the phosphotungstate negative staining technique, they took a number of electron micrographs and compared the particle images to a constructed model. The best representation was concluded to be a capsid composed of 32 morphological sub-units of equal size, arranged in icosahedral symmetry, comprising 12 pentamers and 20 hexamers. These 32 morphological sub-units were composed of 180 identical protein molecules. Considering the molecular weight of each protein molecule as 14,200 (Weber, 1967), this accounts well for a coat protein molecular weight of $2.5 \times 10^6$ calculated from the data of Gesteland and Boedtker (1964). The most recently available data for the amino acid sequence of the coat protein sub-units is the study of Weber and Konigsberg (1967). Their results, using phage f2, indicated that each sub-unit consisted of a polypeptide of 129 amino acids with alanine at the amino terminal and tyrosine at the carboxyl terminal end. There were found to be long stretches of non-polar amino acids; aspartic acid is the only polar residue between

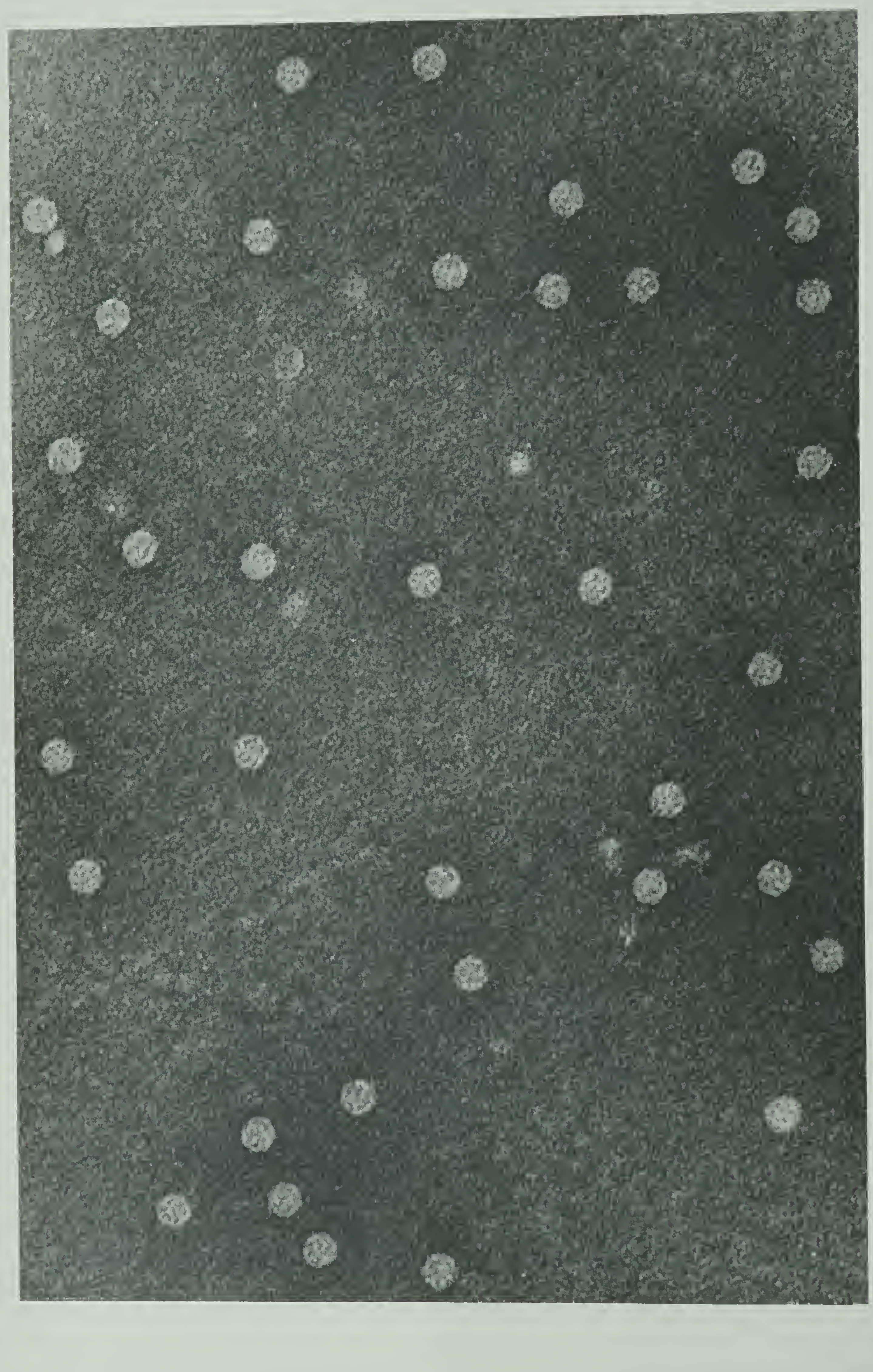


Plate I. Electron micrograph of phage R17

Bacteriophage R17, which had been purified by the normal procedures (outlined in Chapter II), were stained with phosphotungstic acid and examined under the Phillips 200 electron microscope.

X 220,500

residues 1 and 30. It was found that the only difference between f2 and MS2 or R17 was that in the latter case methionine replaces leucine at position 88. Phage fr was found to contain the same number of residues as f2. There were, however, 19 amino acid replacements, 15 of which can be explained by a single base change. It was found that most of the variation occurs at the ends of the molecule and preserves the polar or non-polar nature of the polypeptide sub-unit. In the coat protein of all the RNA coliphages examined, there is a complete lack of histidine detectable by normal protein sequencing methods (see below). In addition, tryptophan is absent from the coat protein of Qβ.

Although it is common to refer to the 180 identical sub-units of the coat protein, there exists evidence, both genetic and physical, to suggest the presence of one, or at most two, sub-units which differ from the other 178 or 179. Certain RNA phage amber mutants, non-infectious because of an inability to adsorb to the host bacterium, have been found to have mutated in the A cistron of the phage genome. This cistron is differentiable genetically from those responsible for the synthesis of coat protein and the induction of a phage specific RNA synthetase (Lodish _et_ _al_., 1965). Nathans _et_ _al_. (1966), using polyacrylamide gel electrophoresis, has separated phage specific proteins from extracts of infected

E. coli and has found four peaks of radioactivity which are apparent in a radioautogram. The four peaks have been characterized as coat protein, an "RNA synthetase", and two minor components which show an incorporated histidine label. Nathans has suggested that the two minor components are in fact a single component and its breakdown product. It is now believed that this minor component is A protein. The finding of this minor fraction in bacteriophage is of importance since it lends physical significance to the nature of the amber mutant in the A cistron. Apparently this A protein is required for adsorption of the bacteriophage since, as previously mentioned, amber mutation in the A cistron gives rise to non-adsorbing phage. Evidence supporting this contention has been presented by Roberts and Steitz (1967). When A protein is added to non-adsorbing (and therefore, non-infecting) phage amber mutants, a small amount of infectivity is restored. Furthermore, in phage reconstruction experiments, these workers have shown that judicious mixing of viral RNA and viral coat protein gives rise to particles which resemble intact phage but which are non-infectious. Addition of A protein to this mixture results in a net conversion of RNA strands to infectious phage particles, although the efficiency of this conversion is very low ($2 \times 10^{-6}$), and reconstituted infectious phage particles are sensitive to

inactivation by ribonuclease whereas normal R17 is not. Presumably, the A protein is the actual molecule through which a phage attaches to its bacterial host receptor site.

The commonly accepted molecular weight for the intact RNA phage is $3.6 \times 10^6$ daltons (Gesteland and Boedtker, 1964). The molecular weight of the coat protein has been shown to be $2.5 \times 10^6$ and therefore, the molecular weight of the RNA is fixed at $1.1 \times 10^6$, which agrees well with the independent physico-chemical determination of the molecular weight of the isolated RNA (Mitra *et al.*, 1963). The RNA content of the phage is therefore approximately 30%. Analysis of the nucleic acid from RNA phage R17 revealed no diphenylamine-reacting material, and the products of alkaline hydrolysis were found to consist of ribonucleotides of adenine, guanine, cytosine, and uracil in the molar ratios 1.00, 1.20, 1.10 and 1.13. No minor base or sugar components were detected (Paranchych and Graham, 1962).

The fact that the RNA is the sole repository of genetic information in the phage has been demonstrated by the infectivity of isolated phage RNA towards spheroplasts prepared from normal male or phage-resistant $F^-$ (female) *E. coli* cells (Davis *et al.*, 1961; Fouace and Huppert, 1962; Knolle and Kaudewitz, 1962; Paranchych, 1963). In the normal infection, there is apparently no significant transfer of

parental protein or nucleic acid to the progeny phage (Ellis and Paranchych, 1963; Davis and Sinsheimer, 1963). The normal burst size in the case of R17 is 10,000 in broth culture; yields in synthetic medium are normally one log lower. A large proportion of the liberated phage, often amounting to 90% of the total, is non-infectious. A fraction of these phage exhibit an anomalous buoyant density in equilibrium density gradients of CsCl, and appear to consist of empty protein shells (Vasquez _et al_., 1966).

The infectious process of RNA bacteriophage can be divided arbitrarily into a number of stages: adsorption of the phage to the host receptor site (the F-pilus), uncoating of the phage nucleic acid, penetration, and replication of the viral RNA. When the viral RNA input of "plus" strand is within the bacterium, it apparently associates with the cell's ribosomes. An enzyme coded for by the phage is produced which catalyzes the synthesis of complementary or "minus" RNA strands. The complex of "plus" and "minus" strands with the enzyme is apparently insensitive to the action of ribonuclease, and is denoted as the replicative intermediate (Weissman, 1967). It is believed that the unaltered "plus" strand acts directly as the template for the synthesis of coat protein, since _in vitro_ synthesis of f2 coat protein material has been reportedly synthesized using isolated

"plus" strands as a messenger RNA (Nathans *et al.*, 1962). Although these replicative steps have been characterized in great detail, there is much less information available concerning the earlier stages of phage infection.

Following the initial report of Crawford and Gesteland (1964) that the RNA bacteriophage R17 attach to filamentous appendages growing from the surface of male strains of *E. coli* K12, it was quickly shown that the other RNA phage also attach to these structures (Brinton *et al.*, 1964; Valentine and Strand, 1965). Brinton has named these thin filaments F-pili, and his group has proposed that F-pili may serve as hollow tubes through which the phage genome is transferred into the host cell, with the possible cooperation of the cell wall or membrane. Filaments analogous to F-pili have been shown to be the apparent adsorption organelles for the *Pseudomonas* phage 7s (Bradley, 1966). The existence of pili, previously called fimbriae, has been known since their description by Anderson in 1949, who described them as fine threads. Other general descriptions of these appendages can be found in the literature (Houwink and Van Iterson, 1950; Brinton *et al.*, 1954; Duguid *et al.*, 1955; Brinton, 1959). They have been found to cause bacterial agglutination, and their adhesive properties may be important in the attachment of bacteria to various nutrients

(Houwink and Van Iterson, 1950). Brinton has been able to identify at least six different kinds of pili which occur on E. coli and Proteus. These pili types are differentiated on the basis of their length, diameter, and frequency, as shown in electron micrographs. Although they are well characterized on the basis of their structures, their functions are not so well understood. Functions have only been suggested for two pili types, Type I and F-pili. The typical Type I pilus has a diameter of 70 Å, varies in length from 0.5 - 2μ, and 100 - 200 such appendages are found on a single cell. The abundance of these pili, and the fact that cells containing only Type I pili can be isolated, has facilitated their characterization. Purified Type I pili, referred to as "pilin", has been shown by chemical analysis to contain no detectable amounts of carbohydrate, lipid or nucleic acid. Over 99% of their weight was found to be due to common amino acids, all as the L isomers. The minimal molecular weight of pilin has been calculated to be 16,600. A large number of amino acids in the molecule have long hydrocarbon chains, and there are relatively few basic amino acids. Brinton (1965) proposed that the large number of non-polar groups could account for the tendency of piliated cells to aggregate and to cause agglutination. Two types of pseudocrystalline aggregates are formed by Type I pili, and the

geometry of these aggregates suggests that the pilus is a rigid helix. This has been confirmed by X-ray diffraction of an oriented wet gel preparation. The pilus has a hollow central core of diameter 20 - 25 Å.

Studies on the genetics and biological function of Type I pili have revealed the following features. The growth and function of the Type I pilus is apparently controlled by a chromosomal gene rather than by an episome (Brinton _et_ _al_., 1961). The colony morphology of piliated and non-piliated cells is sufficiently distinct to allow their differentiation; this feature has made it possible to examine the biological significance of the Type I pilus. Studies on the growth of mixed cultures of piliated and non-piliated cells showed that the population shifts preferentially to the piliated state under conditions of low oxygen tension and high cell density. Whether the growth advantage is due to an increased surface area, or to a specific function of the pili has not yet been determined. It was originally proposed that Type I pili were involved in the transport of bacterial DNA, but Type I-piliation and fertility have been shown to be entirely unrelated.

The observations of Crawford and Gesteland (1964), on the attachment of RNA phage to bacterial cells created an interest in the F-pilus, since clues to its receptor function

were immediately apparent from the electron micrographs. Physically, the F-pilus is a rather striking appendage. It has an average diameter of 85 Å, but unlike the Type I pilus it is not so obviously tubular. Recent micrographs have indicated that an F-pilus can exhibit a tapered appearance (Brinton, personal communication). The implications of this unusual structural feature will have to await further detailed observations. A male E. coli bacterium usually has one F-pilus, although cells with up to four pili can be found. The most dramatic feature of F-pili structure is their extreme length. They can be as long as 20μ, and they often extend outside the field of the electron microscope.

Ørksov and Ørksov, in 1960, demonstrated that male E. coli cells have an antigen which is not present in female cells. A recent report by Ishibashi (1967) suggests that this antigen may be the F-pilus.

F-pili can be prepared by shearing these organelles from the bacteria and removing cells by centrifugation. Various precipitation methods can be used for pili concentrations (Brinton, unpublished manuscript). If F-pili are precipitated in the cold with acetic acid (pH 4), large aggregates form which are completely undissociable by aqueous solvents. These pili are, however, readily dissociated by organic solvents such as chloroform and toluene,

[illegible]

[illegible]

[illegible]

and they are also heat sensitive (Brinton and Beer, 1967). These properties are consistent with the idea that F-pili are composed of lipoprotein molecules. There has been a recent report that F-pilin is sensitive to trypsin, the enzyme giving rise to short thickened rods (Brinton and Beer, 1967). This work has not been repeated, but it seems possible that the pili forms described may have been produced by the incubation conditions rather than by any direct effect of the enzyme. The effect of inhibitors of protein synthesis on the growth of F-pili (Brinton, 1965) lends some support to the premise that the latter are constructed of protein sub-units, although the question is not entirely answered. With certain inhibitors, growth of F-pili is prevented; with others it is not. Brinton has proposed that the lack of inhibition may be the result of growth from preformed sub-units which would be unaffected by inhibitors of *de novo* protein synthesis. F-pili form a single band at a density of 1.19 in equilibrium density gradients of CsCl, but sedimentation in sucrose reveals a heterogeneous population which is consistent with a polymer of repeating structural units (Wendt *et al.*, 1966).

In many electron micrographs of F-pili there is a central dark line which Brinton and Valentine suggest may be due to a central core similar to that found in Type I pili.

This has not yet been further substantiated by X-ray diffraction studies.

Turning from the physical and chemical aspects of F-pili to their biological characteristics, there exists very compelling evidence to suggest that the F-pilus is under the control of the F-episome, the same non-chromosomal DNA that is responsible for bacterial fertility. The growth of the pilus is apparently independent of the state of integration of the episome, since F-piliation occurs in both Hfr and $F^+$ cells. The biological function of these appendages is thought to be the conduction of nucleic acid in both bacteriophage infection and in conjugation, since, unlike Type I pili, F-pili are required for fertility.

Some of the observations which provide support for Brinton's pili conduction hypothesis are the loss of donor capacity of male cells which occurs when their F-pili are removed, and the return of fertility which coincides with pili regrowth. Similarly, the adsorption of RNA coliphages or rod-shaped DNA phages (f1, fd) does not occur with non-piliated cells, and recently, Novotny et al. (1968), have demonstrated that f1 bacteriophage interfere with conjugation.

A demonstration of phage nucleic acid within the F-pilus during infection would provide the ultimate proof of

the nucleic acid conduction hypothesis. This is difficult, however, since it would be almost impossible to determine, autoradiographically, whether grain counts are due to internal nucleic acid, or are contributed by adsorbed bacteriophage particles. Similar difficulties are inherent in the autoradiographic demonstration of DNA transfer during conjugation. It is possible that the F-pilus encloses the F-DNA responsible for its synthesis (Brinton, 1965). According to the pili conduction hypothesis the bacterial chromosome passes through the F-pilus during conjugation. Under conditions designed to label cell nucleates, the F-DNA would also be labelled, and an apparent radioactivity in the F-pilus of conjugating cells would not necessarily be chromosomal DNA. It is clear that, for the present, the F-pili conduction hypothesis will have to stand on circumstantial evidence, which is not completely unequivocal. Recent evidence indicates that in the case of the rod-shaped DNA phage fd, the protein coat label can be transferred to the progeny phage, implying that the phage protein enters the bacterial cell (Trenkner, 1967). The diameter of the fd phage is 60 - 70 Å, while that of the F-pilus is 85 Å. In the case of Type I pili (diam. = 70 Å), the hollow core has a diameter of 20 - 25 Å. If it is assumed that the same diameter to core relationship holds for F-pili, it is difficult to imagine that the core of the F-pilus

would be large enough to accommodate an intact phage. It is possible that the rod-shaped DNA phage infect by passage through the wall of the bacterium, which is the way they are liberated, i.e. extrusion without cell lysis (Hofschneider and Preuss, 1963). If this is the case, then DNA phage infection should not necessarily require a male bacterium, unless the F-factor in some way modifies the entire cell envelope, or unless the phage enters at the base of the F-pilus. It is obvious that this particular aspect will require some clarification.

A large amount of data supporting the idea that F-pili are involved in chromosomal transfer during conjugation has been reviewed in detail by Brinton and Beer (1967) Although the evidence is strongly in favor of the F-pilus playing an active role in the DNA transfer mechanism, other functions for the F-pilus have been proposed. Ippen and Valentine (1967) have suggested that F-pili might provide the hypothesized "mating signal" for conjugation as proposed by Jacob (1966). Brinton and Beer (1967) have indicated that F-pili could act as stabilizers of the mating pair during DNA transfer. In both these cases the loss of donor ability and its return would be affected by shearing of the cells. These additional F-pili functions would certainly extend the capacity of a piliated cell. However, until the

mechanism of conjugation is known in complete detail, these additional F-pili functions tend to obscure the primary conduction role of this appendage.

While the exact function of F-pili in DNA phage infection and in conjugation is still being argued, the role of the F-pilus as an RNA conduction tubule in the early stage of RNA phage infection has been readily accepted on the following evidence. RNA phage attach to these organelles _in vitro_ as well as _in vivo_; bacteria which are genotypically female, and thus phenotypically devoid of F-pili, are resistant to infection by RNA phage; finally, male F-piliated bacteria lose their ability to serve as natural hosts for RNA phage infection when their F-pili have been removed by shearing. Other information which may provide evidence for the participation of F-pili in RNA phage infection is the isolation by Silverman _et al_. (1967), of F-pili mutants of _E coli_ Mutation has been induced by treatment with 100 μg./ml. N-methyl-N'-nitroso-N-nitrosoguanidine (NTG). The mutant cells still produce F-pili to which phage adsorb. Penetration of viral RNA is blocked at low multiplicities, but can be "forced" into the cell at high multiplicity. The "forced" RNA is reportedly non-infectious. Qβ and f1 readily infect the mutant cell, and it has been suggested that this may be a case of a host range mutant (Qβ) in the RNA phages.

The present study has been based on the tacit assumption that F-pili are, in fact, the sites of RNA coliphage attachment and viral RNA penetration. The experiments presented in this thesis represent an attempt to describe, in as complete detail as possible, the conditions under which phage and F-pili associate to form stable complexes, the physiological aspects of the growth of F-pili, the role of the cell's metabolic state in the uncoating of the phage nucleic acid, and the fate of the coat protein during infection.

# II. PREPARATION AND PURIFICATION OF BACTERIOPHAGE R17

## Sterilization procedures

All media and glassware were sterilized for 15 minutes in a steam autoclave at 250$^{o}$F and at a pressure of 18 psi.

Sterile technique was always used in the handling of bacteria and bacteriophage.

## Hard agar (plate agar)

Hard agar, which served as the solid nutrient medium for growth of bacteria, was prepared as follows: To 1 liter of distilled water was added 30 g. of Trypticase Soy Broth (Baltimore Biological Laboratories) and 15 g. Bacto-Agar (Difco Laboratories). This solution, which had a final agar concentration of 1.5%, was autoclaved and dispensed into disposable pre-sterilized petri dishes.

## Soft agar (top agar)

Soft agar was prepared in essentially the same manner as was plate agar, but the final agar concentration was 1.1%. Soft agar was usually prepared on the day of its use, and while hot was pipetted in 1.5 ml. aliquots into sterile culture tubes which were in a 45$^{o}$ water bath.

## Dilution saline

The solution used throughout these studies to dilute either bacteria or bacteriophage, for enumeration by the colony or plaque counting technique, was 0.9% NaCl, containing 5 mM $MgCl_2$ and 5 mg.% bovine serum albumin. The latter component was sterilized separately by filtration through a Seitz asbestos filter and was added to the saline just before its use.

## Minimal medium for the growth of bacteria

The tris (hydroxymethyl) aminomethane-maleic acid minimal salts solution (TMM) had the following composition: 0.05 M Tris, 0.05 M maleic acid, 0.043 M NaCl, 0.027 M KCl, 0.019 M $NH_4Cl$, 0.001 M $Na_2HPO_4$, and 0.001 M $Na_2SO_4$. This medium was adjusted to pH 7.3 with NaOH before autoclaving. Complete or glucose TMM was prepared by mixing 90 ml. TMM salts with 1.0 ml. 50% glucose, 1.0 ml. 0.5% DL-methionine, 1.0 ml. 0.5 M $MgCl_2$, 1.0 ml. Basal Eagle Amino Acid (100x) Concentrate and 5.0 ml. 20 mg.% d-biotin. Minor variations of this medium are described in the text.

## Bacterial strains

*Escherichia coli* $Hfr_1$ K12 $met^-$ ($\lambda^+$) was used as the host bacterium for all studies on bacteriophage infection, and for the preparation of all bacteriophage lysates.

*Escherichia coli* B/r HB11 $F^+$ $lac^+/lac^-$ $str^s$ was the gift of Dr. C. C. Brinton, Jr. This bacterial strain having only F-pili, was used specifically for the preparation of concentrated F-pili stocks. Both bacterial strains were maintained on Trypticase Soy Broth (TSB) agar.

## Enumeration of bacterial cells

(a) *Plate counts*. One ml. of viable bacteria, at the appropriate dilution, was added to 1.5 ml. of soft agar, and the agar-diluent mixture was poured onto a hard agar plate. When the top agar had hardened sufficiently, the plate was inverted and incubated at $37^{\circ}$. Colonies were visible after overnight incubation and each colony was considered to represent one original bacterial cell.

(b) *Spectrophotometric method*. Bacterial cells were grown in broth or complete TMM. At intervals, aliquots were removed, their absorbancies at 650 mμ measured, and cell densities determined by plate counts. From these data, a curve relating $A_{650}$ mμ to cell density was constructed.

## Enumeration of bacteriophage

(a) *Plaque assay*. The plaque assay was employed for the counting of infectious bacteriophage. This consisted of mixing 1 ml. of a known dilution of phage with 0.2 ml. of a broth culture of *E. coli* $Hfr_1$ in mid-log phase

(seed culture), adding 1.5 ml. of top agar, and pouring the mixture onto a hard agar plate, which, after 5 minutes, was inverted and incubated at 37° overnight. Clear areas of lysis (plaques) formed in the lawn of seed bacteria. These were counted and the phage concentration was calculated from the dilution factor, since, theoretically each plaque reflects the addition of one infectious phage particle to the assay plate.

(b) Spectrophotometric determination. Not all virus particles in a given culture are infectious, and it is often of interest to know the ratio of total particles to plaque forming units (PFU's). The total number of particles can be counted in the electron microscope, but a more convenient method is the calculation based on optical density. Using the physicochemical data of Gesteland and Boedtker (1964), we have calculated that a suspension containing $2.18 \times 10^{13}$ particles per ml. has an O.D. 260 mμ of 1.0 [measured in standard saline citrate buffer (SSC)]. SSC = 0.15 M NaCl - 0.015 M sodium citrate.

## Preparation and purification of the bacteriophage R17

A culture of E. coli $Hfr_1$ was grown in a shaking water bath at 37°. When the cell density reached 2 - 4 x $10^8$ cells per ml., based on the $A_{650}$ reading, R17 bacteriophage was added at a multiplicity of infection of 10 - 50

PFU/ml. Incubation was continued for two hours, after which the culture was lysed artificially by the addition of several drops of $CHCl_3$ and an additional 15-minute incubation period. The lysate was chilled, and debris was removed by centrifuging for 10 minutes at 10,000 g. The supernatant was made 5 mg.% with respect to bovine serum albumin (a procedure which minimizes loss due to heavy metal inactivation of phage), and was centrifuged for 4 hours at 27,000 rpm in a number 30 Spinco rotor. The phage pellets, resuspended in 0.1 M Tris buffer (pH 7.3) with a glass pestle or by agitating with glass beads on a vortex mixer, were centrifuged at 10,000 g. for 5 minutes to remove additional debris, and the low speed supernatant, containing the phage, was subjected to an additional cycle of high and low speed centrifugation. It should be pointed out that although the phage theoretically does not sediment at low speed (10,000 g.), much infectivity was often lost in the low speed pellet, and it was often necessary to rewash the pellets several times to extract the phage. The phage suspension, after two cycles of high and low speed centrifugation, was sonicated at 10 kc./sec. for 2 minutes in a Raytheon Model DF101 sonic oscillator, and the sonicate was incubated for 10 minutes at 37° with 5 $\mu$g./ml. RNase to degrade contaminating ribosomes. The phage was chilled, made up to 3.0 ml. with

0.1 M Tris buffer (pH 7.3), and 1.90 g. of CsCl was added. The solution of phage and CsCl was then transferred to an SW39 swinging bucket rotor tube, and an equilibrium density gradient was formed by a 48-hour centrifugation at 35,000 rpm. A single band of phage formed in the middle of the rotor tube, and this was collected from the bottom of the tube by means of a hollow needle. If an unusually large amount of phage were centrifuged in a CsCl gradient, two bands were formed, separated by scarcely a millimeter. The lower band, containing the infectious material (J. Hudson, personal communication), was dialysed versus 0.1 M Tris, pH 7.3, at 4° to remove residual CsCl. With phage solutions having a titre of $1 \times 10^{14}$ PFU's per ml. or less, the addition of 1.90 g. CsCl per 3 0 ml. of phage gave a solution with an initial density of 1.43 g./ml.$^3$. The amount of CsCl had to be decreased, however, to give this same initial density if phage solutions were more concentrated. Yields of the purified phage were normally in excess of 50%, based on the titre of the initial crude lysate.

## Preparation of radioactive R17

Culture volumes were normally limited to 100 ml. for the preparation of radioactive bacteriophage. In the case of $P^{32}$-labelling, the radioactivity (usually 5 mc.), was added as a sterile solution of orthophosphate (obtained

as the di-sodium salt solution from Charles Frosst, Montreal). Bacteriostatic or bacteriocidal agents are often added to radioactive phosphate solutions to prevent contamination, and the amount of one of these chemicals, para-hydroxybenzoate, added in 5 mc. of $P^{32}$-phosphate solution, was sufficient to reduce the yield of bacteriophage by 90%. $P^{32}$ was, therefore, always ordered free of preservatives. Lysates were prepared in complete TMM, which has a phosphate concentration of 1 mM.

In the case of $C^{14}$-labelled and $H^3$-labelled phage preparations, radioactive uracil or a number of different amino acids were used, as indicated in the text (all from Schwartz Biochemicals). In the latter case, the amino acid concentrate was omitted from the culture medium. All radioactivity, $P^{32}$, $C^{14}$ or $H^3$, was added to the culture 10 minutes after the addition of the bacteriophage.

### Properties of the purified bacteriophage

Purified R17 was found to have an $A_{260}/A_{280}$ ratio of 1.84, and to exhibit a single peak in the ultracentrifuge with a calculated sedimentation value of 78S (Figure 2.1). The particle/PFU ratio varied from 4 to 10.

Although a crude lysate of R17 is stable at temperatures below 0°, freezing of a purified suspension of R17 causes a 99% loss of plaque forming units. The phage was

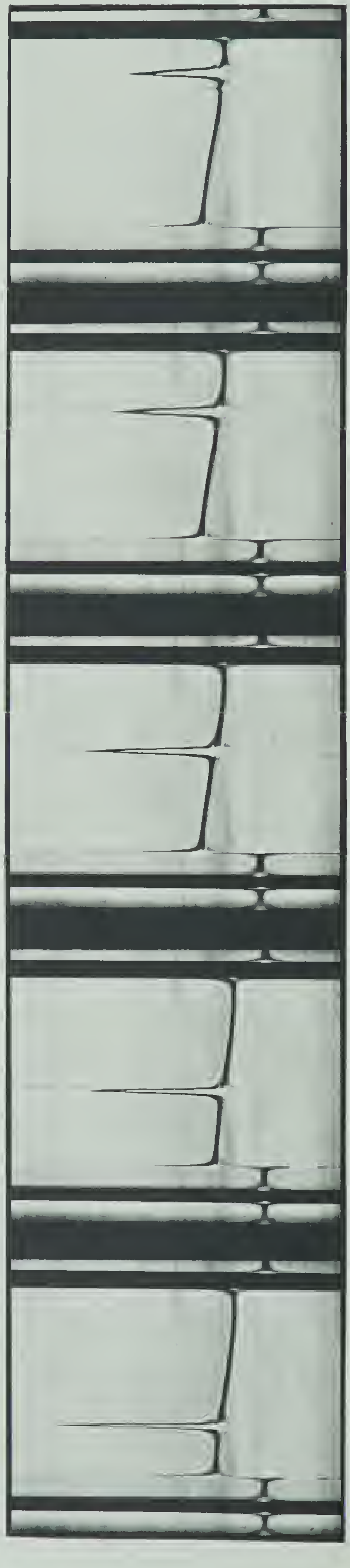


Figure 2.1. Schlieren photographs taken during the course of sedimentation of purified phage R17 in the analytical ultracentrifuge

A solution of purified phage R17, at a concentration of 10 absorbancy units (260 mμ.) per ml. in 0.1 M NaCl - 0.5 M Tris buffer (pH 7.3), was centrifuged at 35,600 rpm in a Beckman Model E. analytical ultracentrifuge. Photographs were taken at 4-minute intervals. Bar angle = 40° (pictures 1 and 2), 35° (pictures 3, 4, and 5). Temperature = 20°.

found to be stable for several hours at room temperature in TMM, 0.1 M Tris or 0.1 M phosphate buffer at pH 7.3. Normally, however, the phage was stored at a temperature of $0^o$ - $4^o$ and, under these conditions, there was negligible loss of infectivity during a six-month period.

Freshly prepared lysates of purified, $P^{32}$-labelled, R17 (normal specific activity 5 x $10^6$ PFU/cpm) showed a single peak of radioactivity and infectivity in linear gradients of 5 - 20% sucrose. A single peak of radioactivity and infectivity was also observed upon recentrifugation of the phage in CsCl.

# III. IONIC REQUIREMENTS FOR THE ATTACHMENT OF PHAGE R17 TO CELL-FREE PREPARATIONS OF F-PILI

## Introduction

One of the important factors influencing the success or failure of a bacterial virus infection is the efficiency with which the virus adsorbs to the host cell. This adsorption is highly dependent on the ionic environment in which virus-cell contact takes place. The most intensive investigations of these ionic requirements have been carried out using the T-series of DNA-containing coliphages. When T-phage were mixed with their normal host in distilled water, no adsorption was found to take place. Furthermore, if $T_1$ or $T_2$ phage were permitted to interact with E. coli B in buffered saline, attachment occurred provided that the pH of the medium was between 5 and 10 (Puck and Tolmach, 1954). Puck et al. (1950), undertook a detailed study of the ionic requirements for attachment of phage $T_1$ to E. coli B. Measuring the attachment velocity of the virus as a function of salt concentration, they found that for divalent cations, maximal velocity was attained at a concentration of $5 \times 10^{-4}$ M. More interesting than the actual salt concentration that promoted the most efficient attachment, was the fact that lower or higher concentrations of divalent ions caused a

decrease in the attachment velocity. These results were interpreted to mean that the virus and its receptor site have charged groups (most likely a preponderance of carboxyl groups which would give the virus and its receptor sites net negative charges at neutral pH) which require neutralization or charge delocalization, by cations in the medium, before attachment can occur. Too great a concentration of cations would result in an excess positive charge at the attachment site, which would again lead to repulsion and, consequently, a decreased attachment velocity.

This group of workers lost favor with some of the current reviewers in the field of virus attachment (e.g. Weidel, 1958) when they attempted to equate phage adsorption to the bacterial cell with adsorption of phage to ion exchange resins (Puck and Sagik, 1953), since it is difficult to believe that ion exchange substances could serve as suitable approximations of the pattern of receptor sites on the bacterial surface. Such a speculative approach to the complex phenomenon of phage attachment could easily cause a reviewer to look upon the data regarding the ionic requirements for phage adsorption with much reservation. It should be remembered, however, that no group has come forth with an alternate hypothesis for the role of cations in virus adsorption which is supported by experimental data. In

addition, not all phages have the adsorption characteristics of the T-series, and differences occur even between the T-even and T-odd phage.

While a comparative study of the ionic requirements for attachment of the RNA bacteriophage to E. coli K12 would have been of practical as well as academic interest, the immediate impetus for such a study came from a different source. Valentine and Strand (1965), using a simple procedure based on the retention of F-pili-phage complexes by membrane filters, had reported that RNA phage readily attach to cell-free preparations of F-pili, and that this attachment required divalent cations with 0.0032 M $Ca^{++}$ giving maximal adsorption. At the same time, Paranchych (1966) was studying the role of divalent cations in the infection of E. coli K12 by phage R17. He found that divalent cations were not required for phage attachment to whole cells, or for the stage of phage infection involving disruption of the coat protein and release of the RNA from the phage particle. Divalent cations were found to be required for the penetration of the viral genome into the bacterial cell. Other data of Paranchych (unpublished) had shown that when $P^{32}$-labelled phage R17 was added to whole cells, almost all of the phage which adsorbs to the cells was actually attached to F-pili growing from the bacteria. These results posed

an interesting paradox; divalent metals were apparently required for phage adsorption to cell-free preparations of F-pili, but not for adsorption to F-pili which remained attached to the bacterial cell.

It was felt that there was sufficient ambiguity in this question to warrant a reinvestigation of the *in vitro* attachment of phage R17 to cell-free F-pili. The technique employed in this study was a modification of the original Valentine and Strand filtration assay. The results of the present study corroborate the *in vivo* data of Paranchych; that is, that attachment of phage R17 to cell-free F-pili has a non-specific ionic requirement that can be satisfied by mono- or divalent cations and that there is no well defined optimal ionic strength such as had been observed for $T_1$. A number of specific ion effects were noticed, especially with trivalent metal ions, and these are discussed in more detail in the text.

## Methods and Materials

### Bacterial and virus strains

Growth and labelling of bacteriophage R17 and its host *E. coli* K12 have been described in detail in the previous section. Phage R17 was always purified by successive cycles of low and high speed centrifugation, followed by

banding in CsCl and dialysis versus 0.1 M Tris buffer (pH 7.3). *E. coli* HB11, a strain having only F-pili, was used for isolation of F-pili.

## Preparation of cell-free F-pili

A 1.5 liter culture of *E. coli* HB11 was grown (with slow shaking in a 37° rotary water bath) to a density of 7 x $10^8$ bacteria per ml. in Trypticase Soy Broth. The culture was chilled in an ice bath and bacteria were sedimented by centrifuging for 10 minutes at 10,000 rpm in a refrigerated centrifuge. The supernatant was discarded and the bacterial pellets were resuspended in a total of 40 ml. of chilled 0.85% NaCl. The resuspended bacteria were blended for 2 minutes in the 70 ml. cup of a Servall Omni-mixer at a powerstat setting of 50, which is equal to a blade speed of approximately 10,000 rpm. The blending procedure was designed to remove pili from the bacterial cells. (The effect of blending is examined in detail in Brinton, 1965; Brinton and Beer, 1967; Novotny *et al*., 1968.) The blended cell suspension was clarified by centrifugation for 10 minutes at 10,000 g., a procedure which sediments the bacteria, leaving free pili in the supernatant. The pili supernatant was dialyzed overnight against 0.01 M Tris buffer (pH 7.3) (Buffer D) in dialysis tubing (3 cm. flat width) which had been boiled several times in demineralized distilled water. The final

F-pili concentration was adjusted to 10 - 12 mg. protein per ml., by the addition of Buffer D based on the ultraviolet absorbance at 260 and 280 mμ. Pili were held in an ice bath until they were assayed for their ability to adsorb phage.

## Filtration assay

Attachment of phage R17 to F-pili was assayed by a filtration technique similar in principle to that of Valentine and Strand (1965), but extensively modified (as described in the text) to conform to our requirements. The basic rationale of the method is that free phage pass through a filter, but, phage particles attached to pili are retained theron. If radioactive bacteriophage are used, then the radioactivity retained by the filter is an index of the extent of adsorption of phage to pili.

All filtration was accomplished under a vacuum of 12 inches of mercury, using a multiple filter box with eight sets of Millipore spring-clamped sintered glass filters on which 2.5 cm. membrane filters were placed. Determination of radioactivity trapped on membrane filters was performed by suspending the thoroughly dried filters in 10 ml. of toluene scintillation fluid (6 g. PPO, 0.5 g. POPOP per liter of toluene), followed by counting in a model LS200 Beckman scintillation spectrometer.

## Results

### (1) The effect of filter composition on the retention of free phage at various salt concentrations

In our initial experiments with the filtration assay, as described by Valentine and Strand (1965), the observation was made that purified free phage was retained by the Schleicher and Schull, type B-6, cellulose nitrate filters in the presence of mono- and divalent salts. When the phage-filter-complexes were washed with 0.15 M NaCl, in Buffer D, more than 25% of the input phage remained trapped by the filter. More serious was the observation that $Mg^{++}$, at concentrations as low as 1 mM, was also sufficient to cause 25% retention of radioactive phage. It should be emphasized that these experiments were performed using phage and cellulose nitrate filters; no pili were involved at this stage. Since a high background radioactivity which varied with salt concentration would preclude the effectiveness of any ionic study, a search was made for a filter which would have little or no affinity for free phage in the presence of mono- or divalent cations. Aliquots of purified radioactive phage, each containing approximately 2 x $10^{12}$ particles, were suspended in 0.01 M Tris buffer (pH 7.3), containing various amounts of NaCl or $MgCl_2$. The phage solutions were passed through the filters to be tested, followed by a wash with 20 ml. distilled water (the wash liquid used in the Valentine

and Strand F-pili assay). Filters were dried and counted and the radioactivity retained by each filter was expressed as a per cent of the radioactivity in the phage suspension. It can be seen in Table I that the Gelman Metricel type GA-6 cellulose acetate filter has remarkably little capacity to adsorb free phage, whereas the B-6 filter was found to retain up to 42% of added phage. Under no conditions did the GA-6 filter retain more than 0.5% of the input phage. This filter was therefore adopted for routine use in subsequent studies on phage attachment to F-pili.

### (2) The effect of the salt composition of the wash solution on dissociation of preformed phage-pili complexes

Valentine's group has been forced to use a distilled water washing solution for assay of pili-phage complexes in order to keep background radioactivity at a minimum. We observed, however, that such a procedure could undermine the basic assay, since it was found that phage-pili complexes were very sensitive to salt concentration, being easily dissociated by exposure to solutions of low ionic strength. It was important, therefore, to insure that phage-pili complexes trapped on the membrane filters would not be dissociated during subsequent washing of the filters to remove free phage. An experiment was thus carried out to determine the effect of salt concentration of the wash solution on the retention of phage-pili complexes by GA-6 filters. F-pili,

TABLE I

The Effect of Salt Concentration on Retention of $P^{32}$-Labelled R17 Phage by B-6 (Schleicher and Schull) and GA-6 (Gelman) Filters

| Moles/l. Salt | Per cent Retention of $P^{32}$-Labelled Phage R17 | |
|---|---|---|
| | B-6 | GA-6 |
| .001 NaCl | 1.8 | 0.1 |
| .010 NaCl | 1.8 | 0.1 |
| .100 NaCl | 25.3 | 0.3 |
| .500 NaCl | 34.3 | 0.5 |
| .001 $MgCl_2$ | 24.7 | 0.2 |
| .003 $MgCl_2$ | 29.5 | 0.2 |
| .006 $MgCl_2$ | 31.5 | 0.2 |
| .010 $MgCl_2$ | 41.8 | 0.2 |

Approximately 20,000 cpm of purified $P^{32}$-labelled phage R17 were suspended in 4.0 ml. of the appropriate salt solution (buffered to pH 7.3 with 0.01 M Tris) and passed through B-6 and GA-6 filters. The filters were then washed with 20 ml. distilled water and counted in a liquid scintillation counter.

suspended in 0.15 M NaCl in Buffer D, were incubated at 37° for 30 minutes with $P^{32}$-labelled R17. Aliquots of 1.9 ml. were passed through separate GA-6 filters, followed by washing with solutions containing various concentrations of NaCl, all similarly buffered. The filters were then counted in the usual manner. The results of the experiment are shown in Figure 3.1. It may be seen that washing the filters with solutions containing little or no salt caused considerable elution of phage from the filters. Since control studies carried out in conjunction with this experiment showed that essentially no free phage was retained by the GA-6 filter, even when high salt was present in the wash solution, it was concluded that the loss of counts which occurred during the washing of the experimental filters indicated the dissociation of already formed phage-pili complexes. When the NaCl concentration of the wash solution was 0.15 M or greater, the retention of radioactivity was maximal, indicating that little or no dissociation of phage-pili complexes occurs in this solution.

On the basis of the foregoing observations, it was decided that the wash solutions should always contain as much salt as the pili-phage incubation mixture, and preferably, the type and amount of salt in the wash solution should be the same as in the incubation mixture.

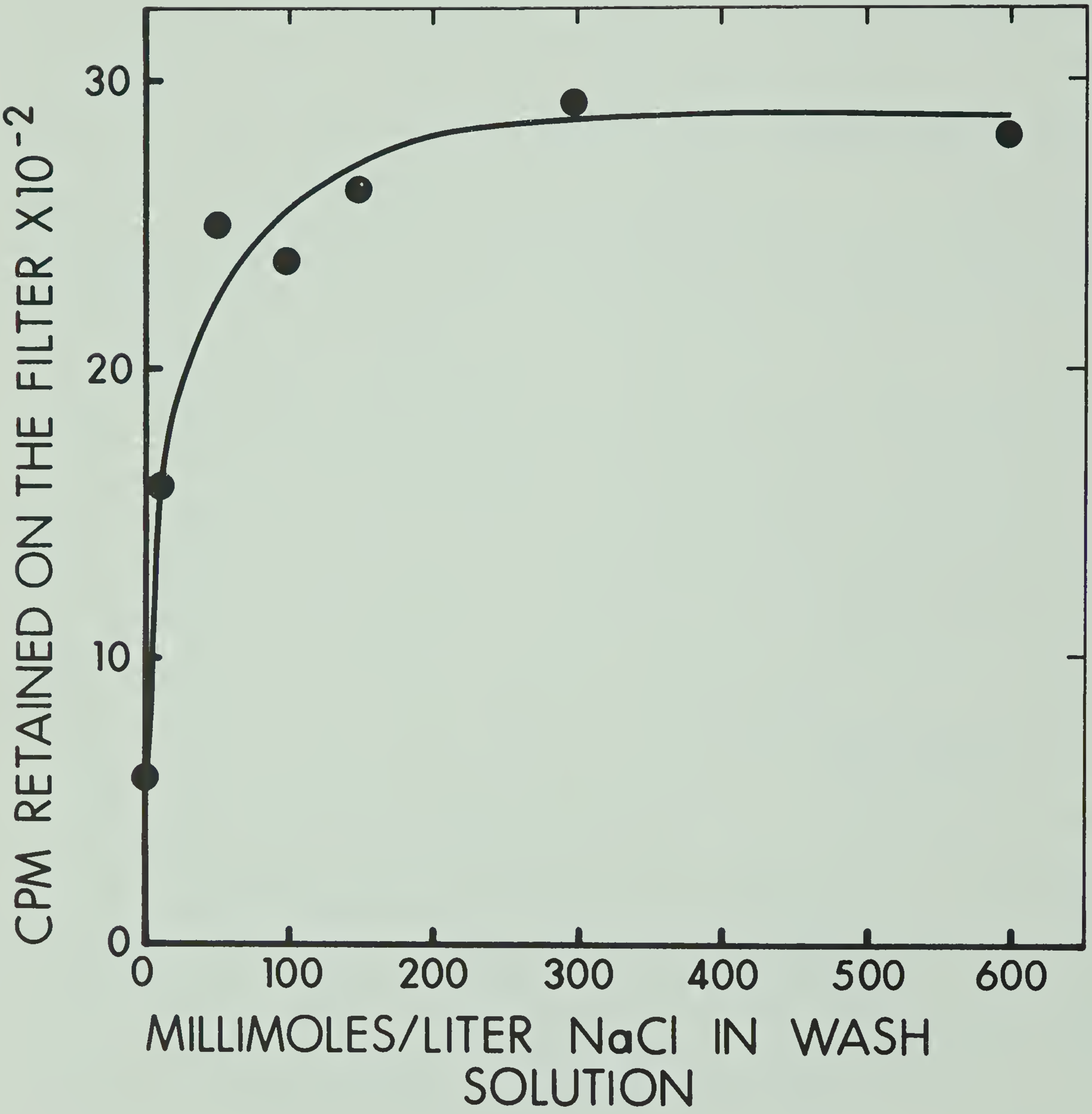

CPM RETAINED ON THE FILTER X10$^{-2}$
30
20
10
0
0
100
200
300
400
500
600
MILLIMOLES/LITER NaCl IN WASH SOLUTION

Figure 3.1. The effect of the salt composition of the wash solution on dissociation of preformed phage-pili complexes

$P^{32}$-labelled R17 (100,000 cpm) and F-pili were incubated for 30 minutes at 37° in 0.15 M NaCl made up in Buffer D. Aliquots of 1.9 ml. of this suspension were filtered on a number of GA-6 filters. Each filter was washed with 20 ml. of Buffer D containing increasing concentrations of NaCl as indicated on the graph. The washed filters were dried and counted in the liquid scintillation counter.

[illegible]

### (3) The stability of F-pili under normal storage conditions

Having established a suitable procedure for the estimation of the formation of phage-pili complexes where the phage-filter interaction was negligible regardless of the type or amount of salt, and where no pili-phage dissociation occurred, it became possible to investigate the specific ionic requirements for adsorption of phage R17 to F-pili. Since the relative extent of attachment was to be compared under various conditions, and since individual sets of experiments were carried out over a number of days using the same pili preparation, it was necessary to develop a standard method for the assay of the capacity of F-pili to adsorb phage. The same preparation of phage was used in any given set of experiments and only required normalization of the radioactivity to a specific date. The standard pili-activity assay solution was 0.5 M NaCl in Buffer D. To 16.6 ml. of this buffer was added 0.24 ml. of stock F-pili, which had been stored in an ice bath, and the mixture was placed in a $37^{o}$ water bath. The reaction was initiated by the addition of $4.8 \times 10^{10}$ $P^{32}$-labelled phage particles (100,000 cpm). Aliquots of 1.9 ml. were removed at various intervals and filtered on GA-6 filters pre-soaked with the same buffer. The filters were then washed with three 5 ml. portions of incubation buffer (room temperature), and the dried filters

were counted for radioactivity. Standard curves were similar in shape to those of Figure 3.3. The 40-minute values of a series of these curves were examined as a function of days after pili isolation to give some indication of the stability of this preparation (Figure 3.2). It can be seen that the activity of the F-pili (as represented by $P^{32}$-labelled phage adsorption) remained virtually unchanged over a period of six days. This was the longest period that a single preparation of F-pili was stored before use. Other experiments have indicated that F-pili are stable at -20°, -40°, and -60°, and in half-saturated ammonium sulfate (pH 7.3) at 0°, with the provision that in all cases the protein content of the pili solution exceeds 0.4 mg./ml.

### (4) The standard assay for the determination of the ionic requirements for the attachment of phage R17 to F-pili

The assay employed for the determination of the effect of various ions on the attachment of phage R17 to free F-pili was essentially the same as the standard pili activity assay.

The standard reaction mixture consisted of 8.3 ml. of the appropriate concentration of a metal chloride made up in 0.01 M Tris buffer (pH 7.3), and 0.13 ml. of F-pili stock. The suspension was warmed to 37° and $2.4 \times 10^{10}$ $P^{32}$-labelled phage particles were added. Aliquots of 1.9 ml. were filtered at 10, 20, 40 and 60 minutes after phage addition, and

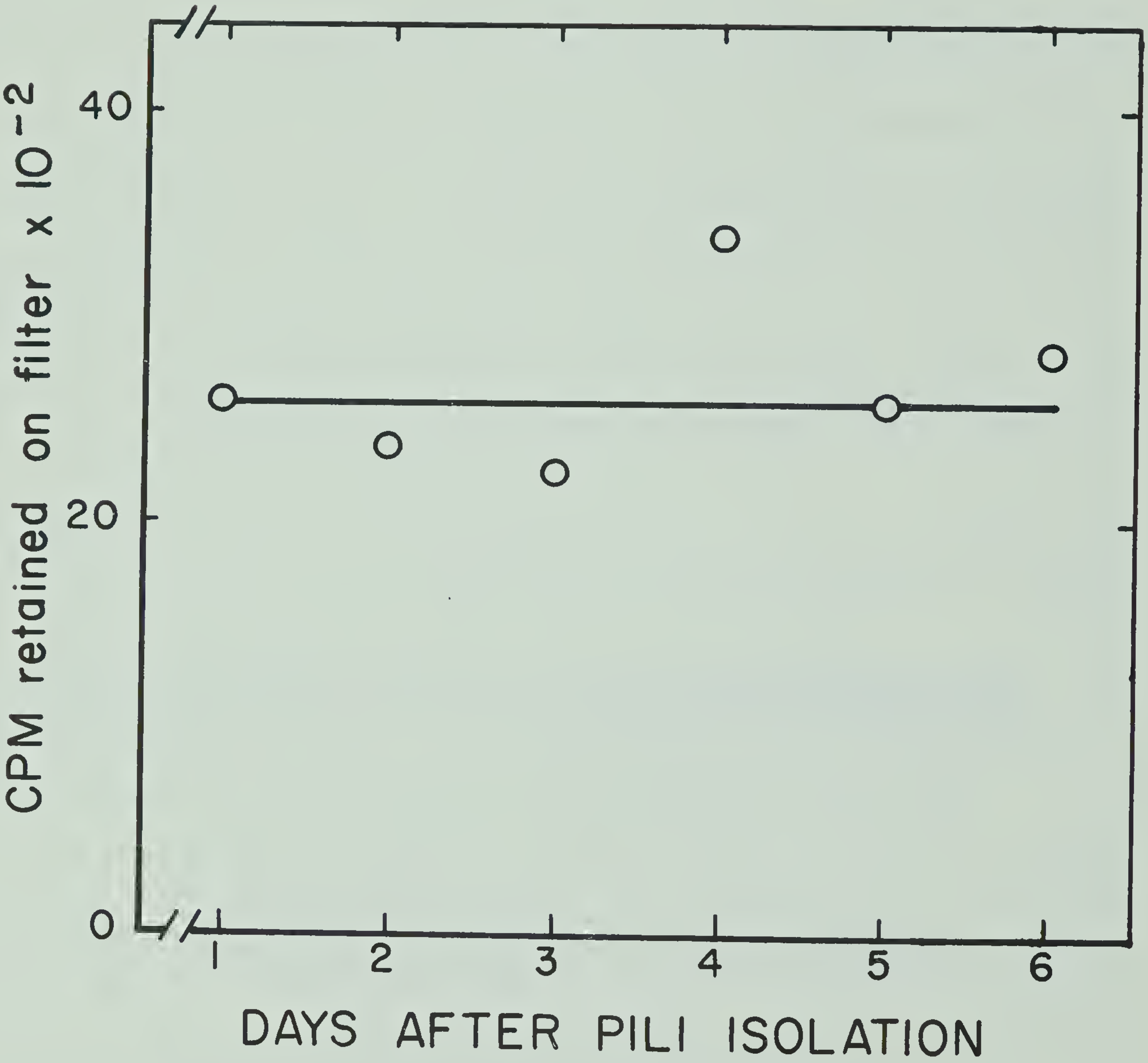

CPM retained on filter x $10^{-2}$
40
20
0
1
2
3
4
5
6
DAYS AFTER PILI ISOLATION

Figure 3.2. The effect of storage in ice on the ability of F-pili to adsorb phage

An aliquot of F-pili (0.24 ml.) was removed from the F-pili stock being stored in an ice bath and was added to 16.6 ml. of 0.15 M NaCl in Buffer D. The mixture was warmed to $37^{o}$ and $4.8 \times 10^{10}$ $P^{32}$-labelled phage particles were added. Aliquots of 1.9 ml. were filtered on GA-6 filters at various intervals over a 60-minute period. The dried filters were counted as usual and the 40-minute value for each adsorption curve is plotted versus day after pili isolation.

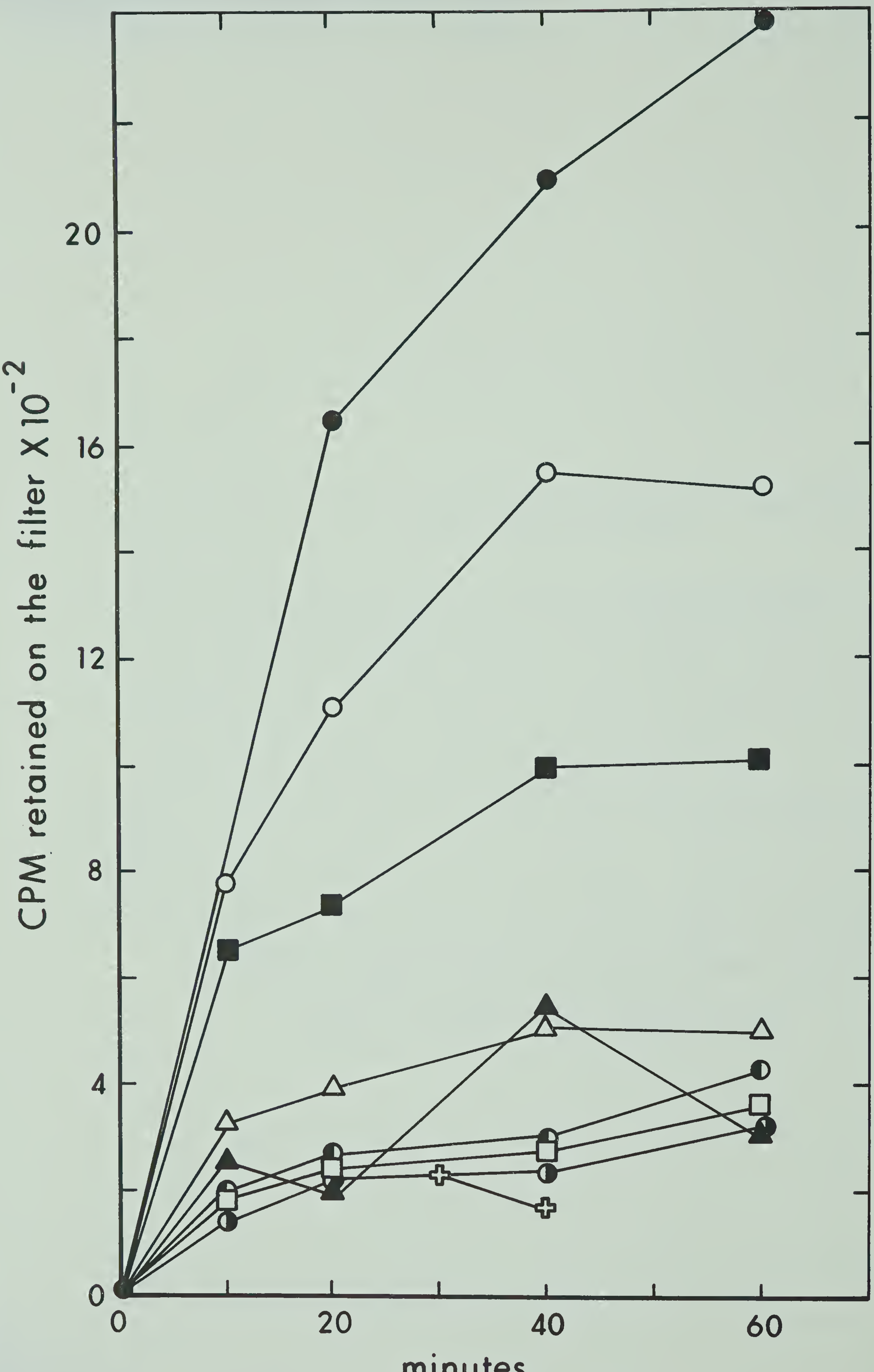

CPM retained on the filter X 10$^{-2}$
0
4
8
12
16
20
0
20
40
60
minutes ,

Figure 3.3. The effect of increasing concentrations of KCl on the attachment of R17 bacteriophage to F-pili

Aliquots of F-pili stock (0.13 ml.) were added to 8.3 ml. volumes of solutions containing various concentrations of KCl in 0.01 M Tris buffer (pH 7.3). The pili suspension was warmed to 37°, and 2.4 x $10^{10}$ $P^{32}$-labelled phage particles were added to each mixture. Aliquots of 1.9 ml. were filtered at 10, 20, 40, and 60 minutes after phage addition, and the filters were washed with three 5 ml. aliquots of the appropriate incubation solution. The filters were finally dried and counted as previously described. 0.01 M Tris buffer (+); 0.1 mM KCl (◉); 0.5 mM KCl (▲); 1 mM KCl (□); 5 mM KCl (◐); 10 mM KCl (△); 50 mM KCl (■); 100 mM KCl (o); 500 mM KCl (●).

[illegible]

the filters were washed with three 5 ml. aliquots of the incubation solution. The filters were finally dried and counted as previously described. The results for each set of cations studied are discussed separately, while general conclusions are discussed at the end of this section of the thesis.

(a) The effect of monovalent cations. $Na^+$ and $K^+$, the two most important physiological ions, were used as representative monovalent cations. In addition, $NH_4^+$ was investigated since it was present in significant amounts in the minimal medium used for the growth of the bacteria. Figure 3.3 shows a complete set of adsorption curves for KCl. It can be seen that the Tris buffer, even at 10 mM (the concentration used in all the incubation mixtures), contributes little to the enhancement of phage adsorption. In the case of KCl, there is a progressive increase in adsorption with increasing concentration above 1 mM. Phage adsorption appears to level off by 10 minutes at the lower salt concentrations, while adsorption in 500 mM KCl does not appear to be complete, even after 40 minutes (the 60-minute point of this curve may be slightly high due to filtration error [see below]). In subsequent experiments, using various cations, similar adsorption curves were obtained, but for purposes of comparison, the data are plotted

in Figures 3.4, 3.5 and 3.6 as the counts retained after a 40-minute incubation period versus the concentration of the salt in the incubation medium. Figure 3.4 shows the results for the three monovalent ions. It can be seen that all three monovalent cations readily promote phage attachment, although the efficacy of the ions was $Na^+ > K^+ > NH_4^+$.

At this point some comment must be made to explain the marked deviation of the value for counts retained after a 40-minute incubation period in 0.5 mM KCl (see Figure 3.3) from that expected on the basis of values at other time intervals. Inherent in most filtration assays is the occasional occurrence of large deviations, points which lie far above or below what are otherwise very regular curves. These are possibly due to imperfect filters which are too thin or too thick, of irregular pore size, or improperly wetted (air pockets trapped beneath the filter). Such values have been rejected in compiling the data presented herein.

(b) The effect of divalent cations. The divalent ions chosen for this study were $Mg^{++}$, $Ca^{++}$, $Mn^{++}$, and $Ni^{++}$. Figure 3.5 shows the effects that these ions have on phage adsorption. $MgCl_2$, $CaCl_2$, and $MnCl_2$ all show superficially similar enhancement of phage attachment to F-pili, although individual differences are apparent, with $Mn^{++}$ and $Ca^{++}$ giving a greater response than $Mg^{++}$. $NiCl_2$ caused an

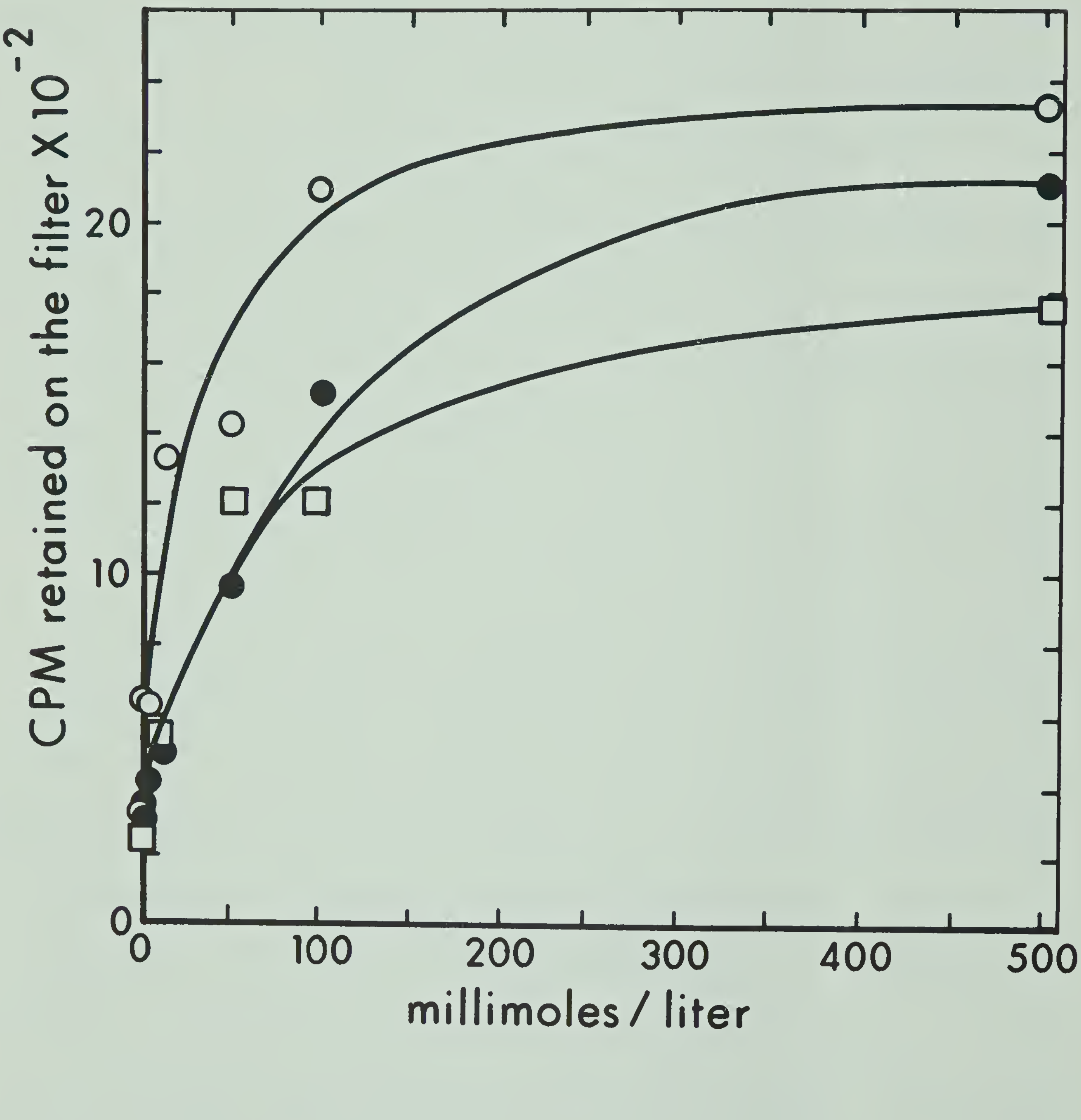

CPM retained on the filter X 10$^{-2}$
0
10
20
0
100
200
300
400
500
millimoles / liter

Figure 3.4. The effect of monovalent cations on the attachment of R17 bacteriophage to F-pili

Details of the procedure followed in this experiment are described in the legend to Figure 3.3. The 40-minute values from each of the curves, relating counts retained on the filter to time of incubation, are plotted here as a function of the concentration of salt in the incubation media. $NH_4Cl$ (□); KCl (●); NaCl (o).

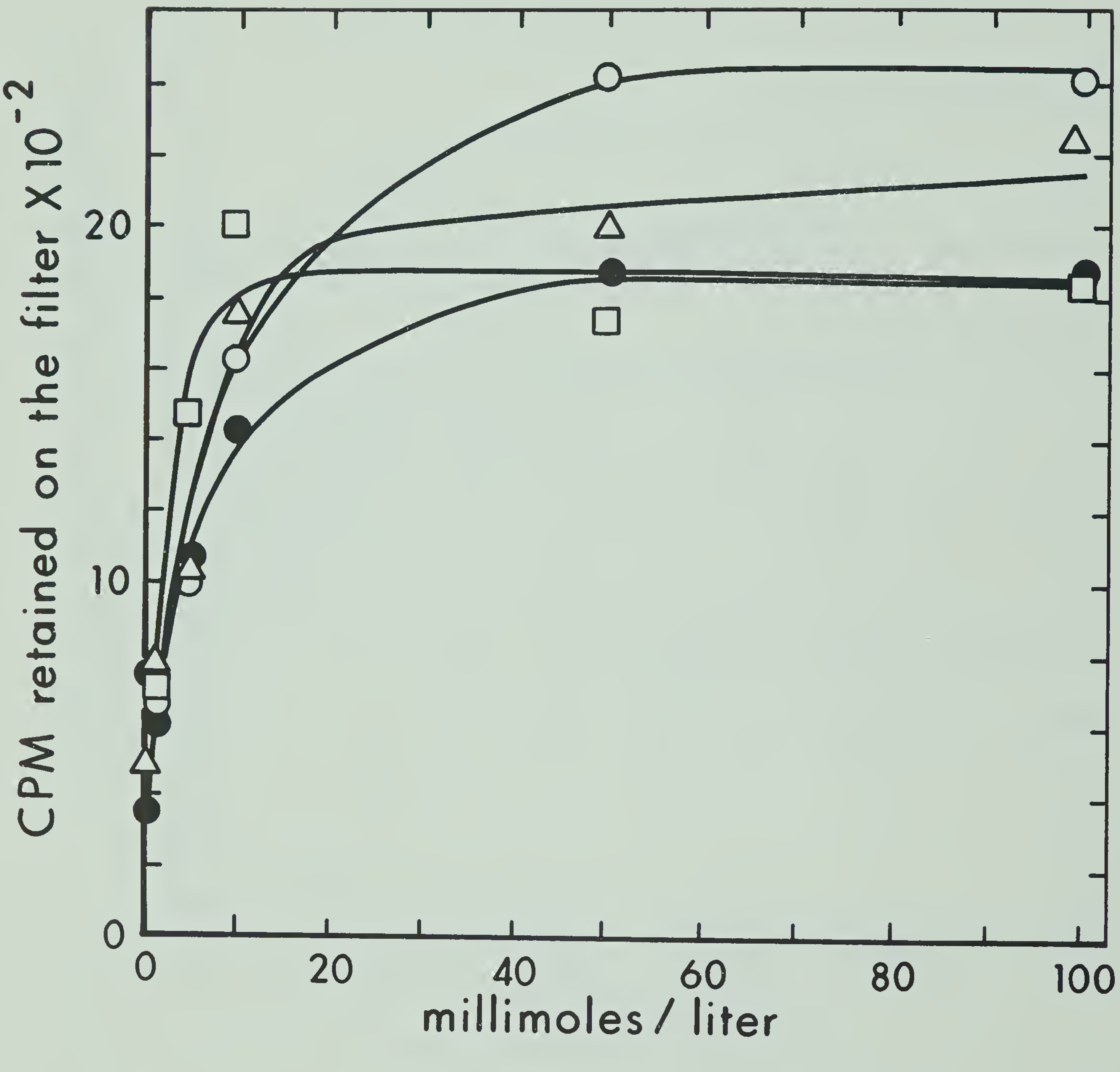

CPM retained on the filter X $10^{-2}$
0
10
20
0
20
40
60
80
100
millimoles / liter

Figure 3.5. The effect of divalent cations on the attachment of R17 bacteriophage to F-pili

Details of the procedure followed in this experiment are described in the legend to Figure 3.3. The 40-minute values from each of the curves, relating counts retained on the filter to time of incubation, are plotted here as a function of the concentration of salt in the incubation media. $NiCl_2$ (□); $MnCl_2$ (o); $CaCl_2$ (△); $MgCl_2$ (●).

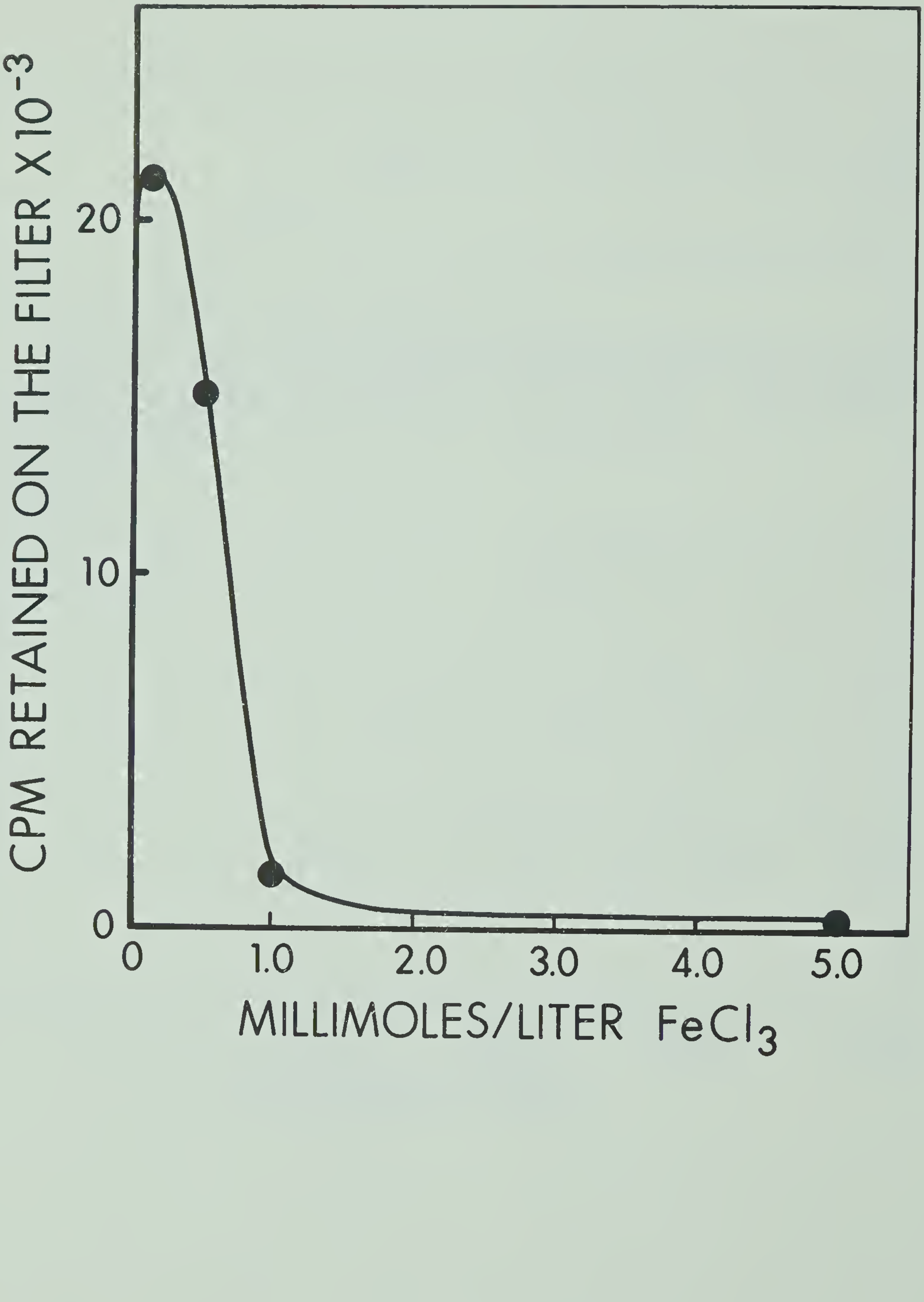

CPM RETAINED ON THE FILTER X10$^{-3}$
20
10
0
0
1.0
2.0
3.0
4.0
5.0
MILLIMOLES/LITER $FeCl_3$

Figure 3.6. The effect of $FeCl_3$ on the attachment of R17 bacteriophage to F-pili

Details for this experiment are described in the legend of Figure 3.3. Due to difficulties described in the text, only 0.1, 0.5, 1.0, and 5.0 mM $FeCl_3$ was used to plot time curves. The 40-minute values of these curves are plotted versus $FeCl_3$ concentration.

extremely efficient stimulation of adsorption when present in the incubation mixtures at levels as low as 10 mM. This, however, appears to be the optimal concentration of $Ni^{++}$, since further addition of the salt did not increase the level of phage attachment.

(c) The effect of trivalent cations. The most widely known biologically occurring trivalent ion is the ferric ion which participates in many oxidation-reduction reactions in living tissue. It occurs bound to enzymes and bound to the porphyrin ring of hemoglobin. Other trivalent cations, if they exist naturally in living organisms, are likely to be present only in trace amounts. As a representative sample of these other trivalent ions, $Al^{+++}$ and $Cr^{+++}$, both as the chloride salt, were tried in addition to $Fe^{+++}$. It was felt that if ions stimulate the adsorption of phage by neutralization of charge, or by producing a critical net charge necessary for the proper conformation of the attachment site, trivalent ions should act at an extremely low concentration since they combine a large charge with a small ionic radius. Figure 3.6 shows the results for $FeCl_3$. The results obtained with the other ions were quite similar to those shown in Figure 3.6. It can be seen at once that these ions do not act in proportion to their concentration. It was found that those filters which had high counts also

had a slow filtration rate, and the filter had a glassy sheen, which was colored or colorless, depending on the ion used. Close examination of the trivalent cation incubation mixtures, with or without phage, revealed a flocculent precipitate which formed on standing either at room temperature, or at 37°. This precipitate was not dissociable by chloroform or toluene, which made it difficult to determine whether the precipitate involved F-pili or whether it was a metal complex induced by pili. It must be further noted that although these pili were purified, they probably contained some impurities. This question has not been satisfactorily resolved. If, however, the complex is of F-pili, this could provide the basis for a method of selective precipitation which could be used as an adjunct to existing techniques for the concentration and purification of pili.

(d) The action of mono- and divalent cations as a function of ionic strength. The salt concentrations employed in the various sets of experiments have been reported in the previous sections as moles per liter. There is, however, a common expression which is independent of the specific salt used; this is the ionic strength, $\mu$, which is defined as: $\frac{1}{2} \sum c_i Z_i^2$ where $c_i$ is the molar concentration of the $i^{th}$ component and $Z_i$ is its charge. When the data for NaCl and $MgCl_2$ were plotted in this manner, the curve of Figure 3.7

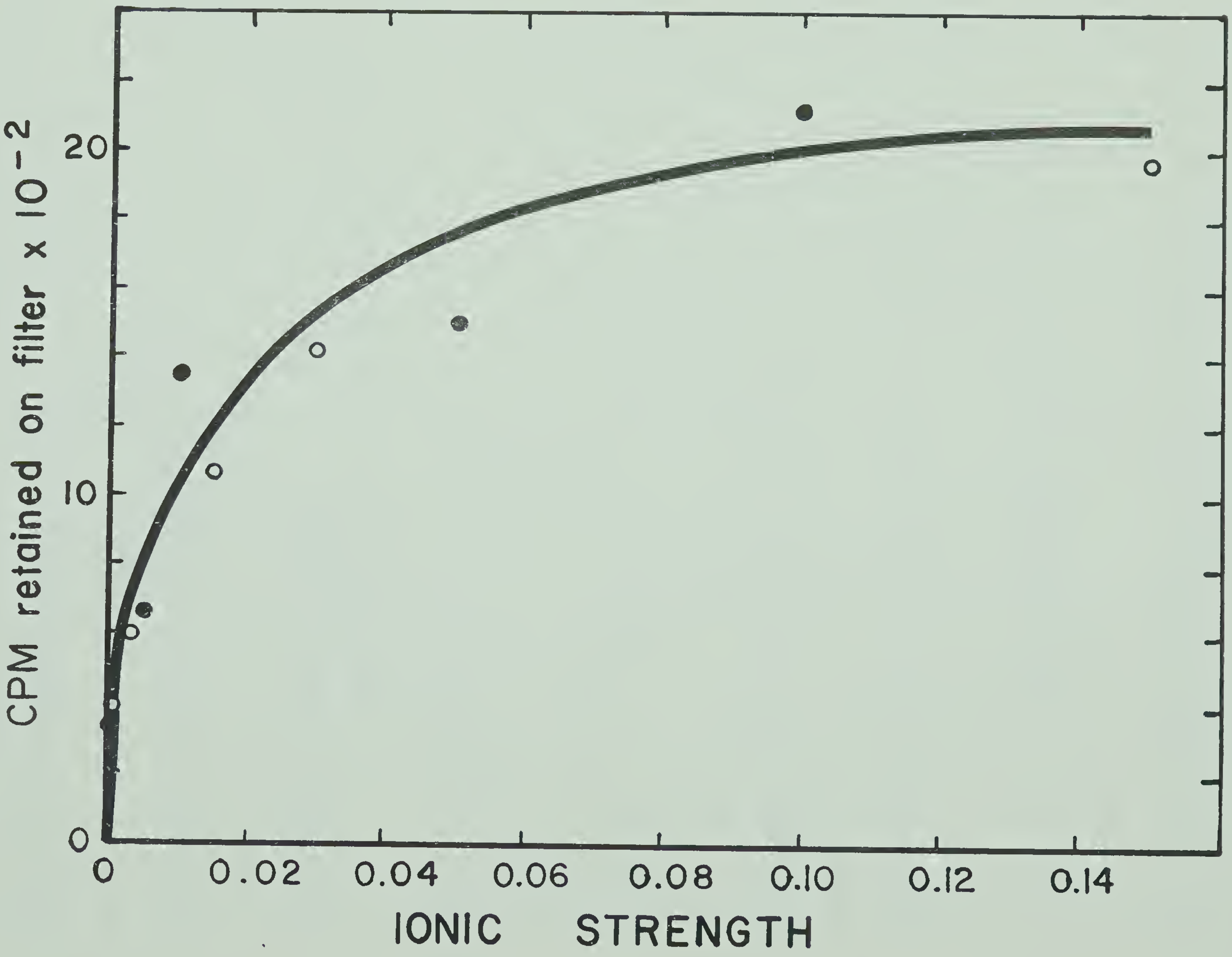

CPM retained on filter x $10^{-2}$
0
10
20
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
IONIC STRENGTH

Figure 3.7. Adsorption of R17 to F-pili as a function of ionic strength of the incubation medium

The data illustrated in Figures 3.4 and 3.5 are replotted here, with counts retained on the filter expressed as a function of ionic strength. $MgCl_2$ (o); NaCl (●).

was obtained. The implications of these results will be considered in the discussion section of this chapter.

(e) The effect of mixed cations on the adsorption of phage. The next logical step after looking at monovalent and divalent ions separately was to examine the effects of combinations thereof.

The experiments described in what follows were of two types. Firstly, concentrations of monovalent cations giving maximal stimulation were mixed with sub-optimal but stimulatory levels of the various divalent cations, and these solutions were employed as incubation media in assays of phage-pili interaction. In the second case, solutions containing sub-optimal levels of both mono- and divalent cations were employed in the assays, to determine whether or not the stimulatory effects of the two ionic species are additive (Figure 3.9).

The addition of various divalent ions, at a final concentration of 10 mM, to solutions containing the optimal level of NaCl, did not produce any stimulation of phage-pili interaction over and above that observed in the solution containing only NaCl (Figure 3.8).

Figure 3.9 shows time-course curves of phage attachment in the presence of 10 mM NaCl ($\mu$ = 0.01), 5 mM $MgCl_2$ ($\mu$ = 0.015), and a mixture of the two ($\mu$ = 0.025). Reference

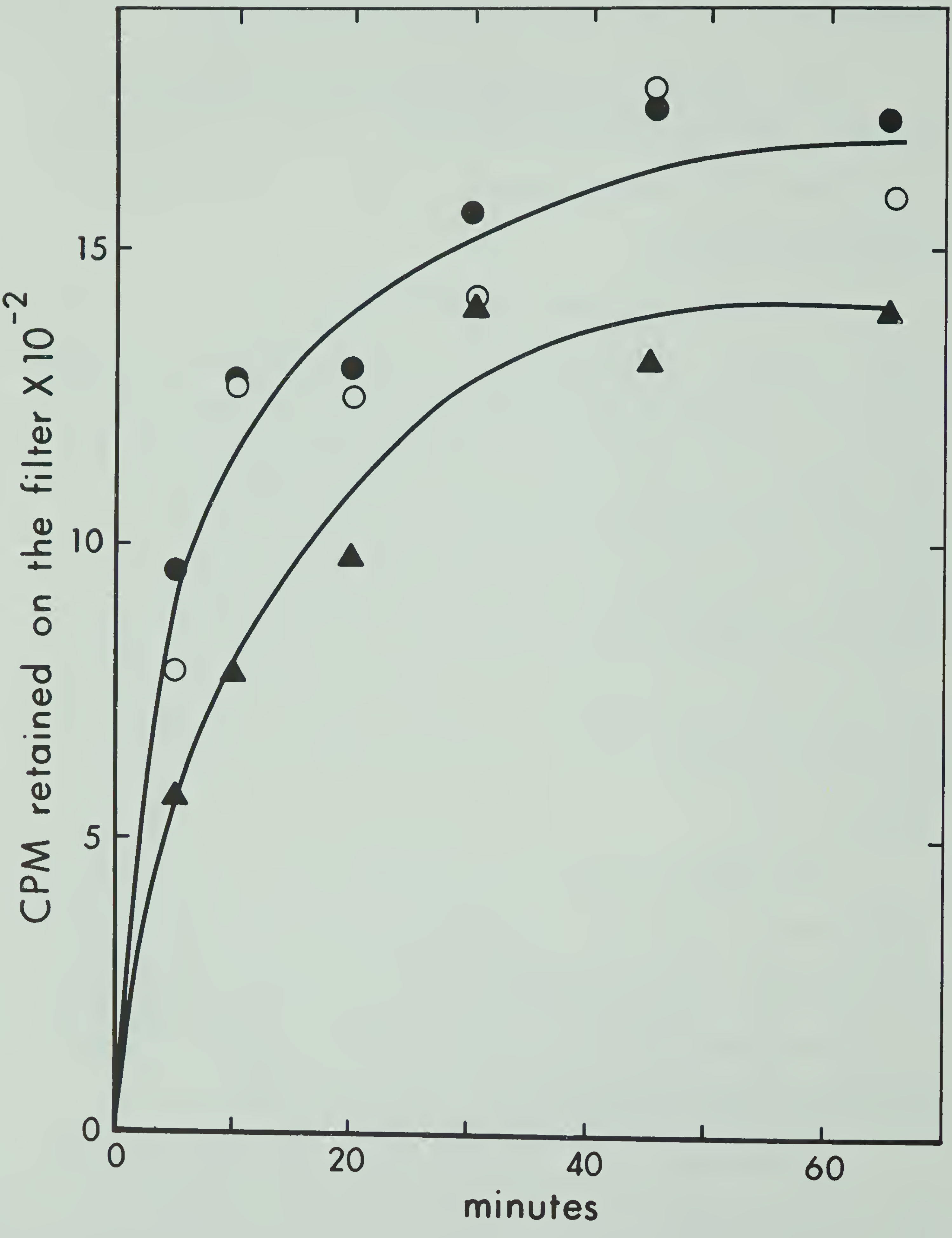

CPM retained on the filter X $10^{-2}$
0
5
10
15
0
20
40
60
minutes

Figure 3.8. The effect of sub-optimal amounts of divalent cations on the adsorption of R17 bacteriophage to F-pili in the presence of optimal levels of NaCl

The experimental procedure is as described in Figure 3.3. The volume of the incubation mixture was 12.0 ml., consisting of 11.8 ml. of the mixed salt solution and 0.2 ml. of F-pili. Phage input was $4.8 \times 10^{10}$ $P^{32}$-labelled particles and filtration times were as indicated. 0.5 M NaCl - 10 mM $NiCl_2$ (▲); 0.5 M NaCl - 10 mM $MgCl_2$ (○); 0.5 M NaCl (●).

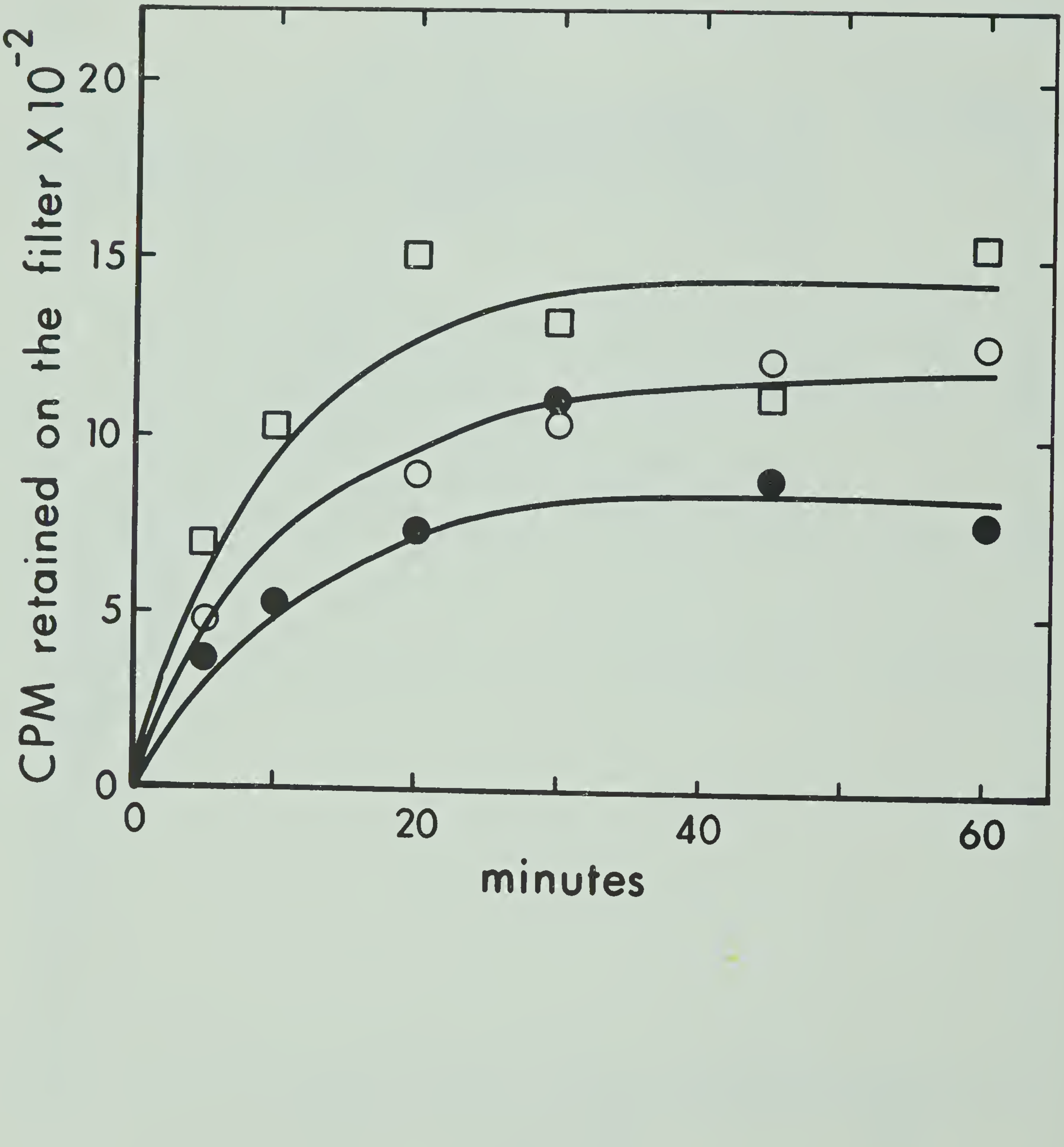

CPM retained on the filter X 10$^{-2}$
0
5
10
15
20
0
20
40
60
minutes

Figure 3.9. The effect of sub-optimal amounts of mixed mono- and divalent cations on the adsorption of R17 bacteriophage to F-pili

The experimental procedure is as described in Figure 3.3, with the additional modifications as outlined in Figure 3.8. 10 mM NaCl (●); 5 mM $MgCl_2$ (o); 10 mM NaCl - 5 mM $MgCl_2$ (□).

to Figure 3.7 shows that the effects on these ions are additive, since the amount of phage attachment occurring in the presence of the mixture ($\mu = 0.025$) is the same as is found with either salt alone when it is present at an ionic strength of 0.025.

(f) The effect of sucrose on the attachment of phage to F-pili. The effect of sucrose on phage adsorption to F-pili was examined to investigate the possibility that stimulation of phage attachment by cations was a general effect of increased osmolarity of the incubation mixtures, rather than a specific effect of electrolytes. Equivalent amounts of F-pili were diluted into sucrose solutions (in Buffer D) of various concentrations at $37^{o}$, and, to each sample an aliquot of a suspension of $P^{32}$-labelled phage was added. After 20 minutes, the contents of each incubation mixture (total volume 2 ml.) were pipetted onto GA-6 filters which were washed, dried, and counted in the usual manner. There was found to be no increase in phage adsorption (over that observed in Buffer D alone) caused by concentrations of sucrose from 0.01 to 0.5 M. Thus, enhancement of R17 attachment to F-pili depends upon electrolytes and is insensitive to osmolarity *per se*.

### (5) The physical state of F-pili during phage adsorption

The kinetics of phage-pili interaction, as measured by filtration, suggest that phage retention on the filters is not due to trapping of particles by a matted and impermeable network of F-pili strands, but is a specific phage-pili attachment phenomenon. F-pili, nevertheless, can associate under certain conditions, and it was of interest to examine the effect that this association might have on the filtration assay. Considering that phage attach along the entire surface of an F-pilus, if F-pili were to aggregate as side-to-side strands, the number of binding sites for phage adsorption would decrease, since portions of two pili surfaces would be unavailable for phage binding. Dissociation of aggregated F-pili preparations, on the other hand, would free sites to which phage could then adsorb. It was therefore important to demonstrate that the adhesive properties of the F-pili (i.e. the tendency to aggregate) are not affected by the salt concentration or composition of the media used in these studies.

The most logical way to examine this question would have been by means of a rigorous electron microscopic inspection of F-pili exposed to solutions of various ionic compositions and strengths. There were, however, two major obstacles to such a scheme. The first objection was that

too high a salt concentration on the electron microscope grids could completely obliterate the specimen image. The second, and more important obstacle, was the absence of a departmental microscopist and a microscope.

It was necessary, therefore, to devise an alternative method of examining this question, and we turned to the use of sucrose gradient sedimentation analysis. It was reasoned that the random population of F-pili would exhibit a reproducible sedimentation profile in sucrose gradients. If the state of aggregation of the F-pili were dependent upon salt concentration and composition, then the F-pili sedimentation pattern in gradients containing various concentrations of mono- and divalent cations should be altered. F-pili would sediment towards the bottom of the tube in the case of aggregation, and they would remain near the top of the gradient in the case of disaggregation. F-pili detection could be accomplished by testing the phage adsorbing properties of each gradient fraction. It must be assumed that a small fraction of the pili preparation composed of pili fragments can probably pass through the filter.

The experiments outlined above required the use of concentrated F-pili. Wendt _et_ _al_. (1966) had demonstrated that F-pili would band in CsCl at a density of 1.19, and this was the concentration procedure used. To 12.0 ml. of

stock F-pili, prepared in the normal manner, was added 3.0 g. CsCl. The pili-CsCl solution was dispensed into three centrifuge tubes and the gradient was established by centrifuging the tubes for 36 hours at 35,000 rpm. Fractions of 12 drops each were collected from each of the gradients (by means of a hollow needle inserted into the bottom of the tube), and each fraction from one of the gradients was assayed for F-pili in the following manner. Two ml. 1.0 M NaCl and a fixed amount of $P^{32}$-labelled R17 were added to each fraction, and after 8 minutes of incubation at $37^{\circ}$, the contents of each tube were filtered on a GA-6 filter. The filters were washed with two 5 ml. aliquots of 1.0 M NaCl, dried, and counted in the liquid scintillation counter. The results of these assays can be seen in Figure 3.10. The F-pili, as expected, were found to form a single band in the CsCl gradient. The corresponding peak fractions from the other gradients were pooled and dialyzed against Buffer D to remove the CsCl. An electron micrograph was taken of this preparation and is shown in Plate II. It should be noted that although several small pili bundles are present, there are many single strands, which make it reasonable to believe that this material has not been severely aggregated or dissociated by the gradient purification.

The sedimentation properties of these concentrated and partially purified F-pili were examined in linear 18 -

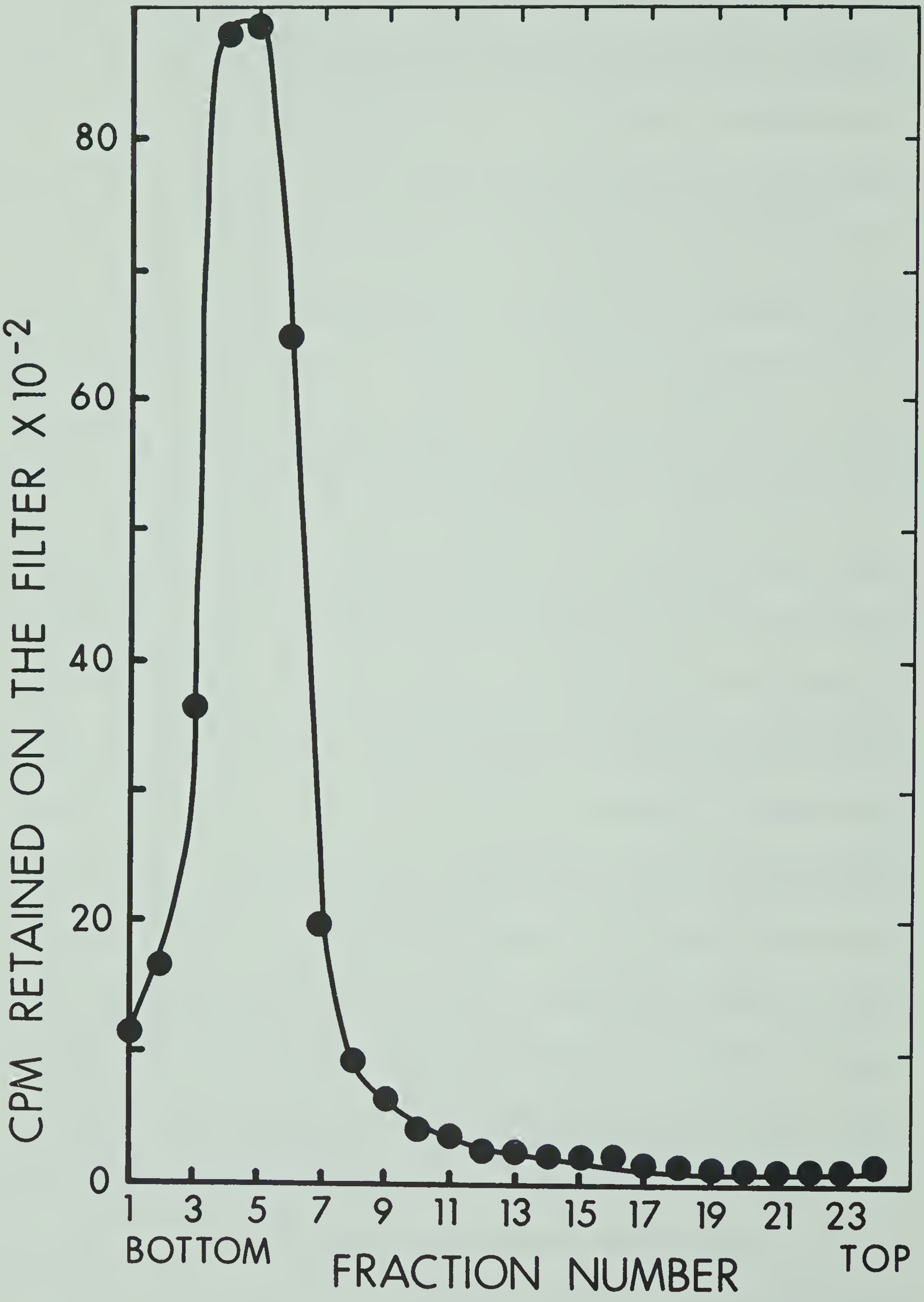

CPM RETAINED ON THE FILTER X 10$^{-2}$
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
23
BOTTOM
FRACTION NUMBER
TOP

Figure 3.10. Cesium chloride equilibrium density gradient sedimentation of F-pili

Three grams of CsCl were added to 12.0 ml. of F-pili stock in Buffer D. The suspension was divided equally among three SW39 gradient tubes, and the gradient was established by centrifugation for 36 hours at 35,000 rpm. Fractions of 12 drops each were collected from the gradients. Each fraction from one of the tubes was assayed for F-pili, as described in the text. The peak fractions of the other two tubes were pooled and dialyzed against Buffer D to remove CsCl.

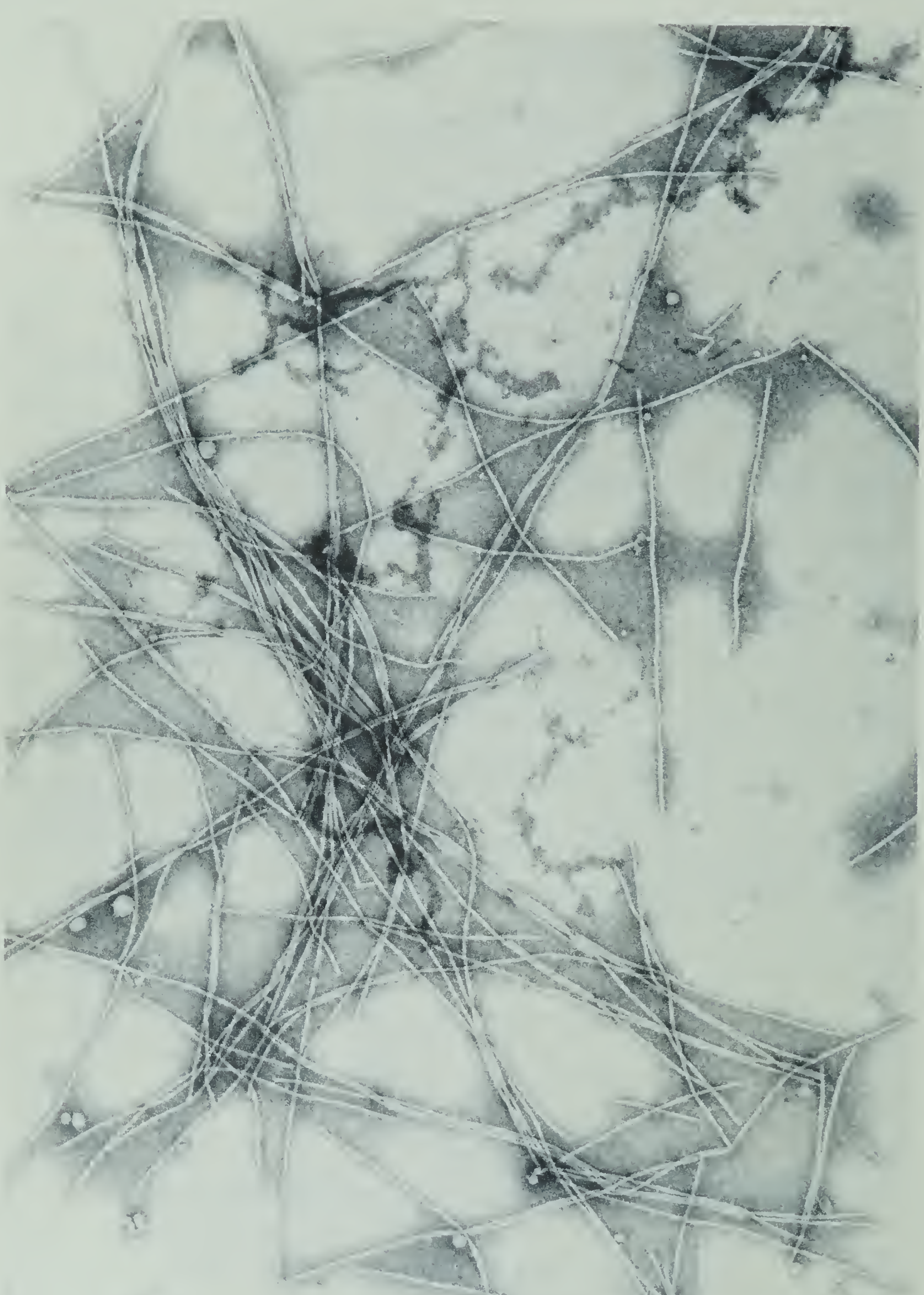


Plate II. The appearance of CsCl concentrated F-pili under the electron microscope

F-pili were prepared as described in the text. One-tenth of a ml. of F-pili was mixed with 0.1 ml. 5% phosphotungstic acid (PTA). Twenty lambdas of this pili-PTA solution were placed on a formvar-coated grid and allowed to dry. The preparation was then examined under the electron microscope. X 127,000.

36% sucrose gradients prepared in the salt solutions indicated in the text figure. Centrifugation was for 3 hours at 35,000 rpm in the SW39 rotor. All gradient fractions were assayed as previously described for the CsCl gradient, and the results are shown in Figure 3.11. It can be seen that F-pili sediment as a fairly broad peak. This is as would be expected, since the pili concentrate contains strands of differing lengths. The main observation to be noted is that the sedimentation properties of the pili did not change significantly in the various salt solutions. According to the original hypothesis, this would mean that F-pili do not associate or dissociate in response to changes in the ionic environment. Thus the results obtained by measuring the effects of cations on the attachment of phage to pili can be regarded as reflecting their effect on phage-pili interactions, rather than on pili-pili interactions.

## Discussion

A membrane filtration assay was used to determine the effect of various ions on phage adsorption to F-pili.

Cellulose nitrate filters were found to bind free bacteriophage, depending on the amount of salt in the suspending medium. These filters were discarded in favor of a cellulose acetate filter (Gelman GA-6) which retained less than 0.6% of the input phage. The results of experiments

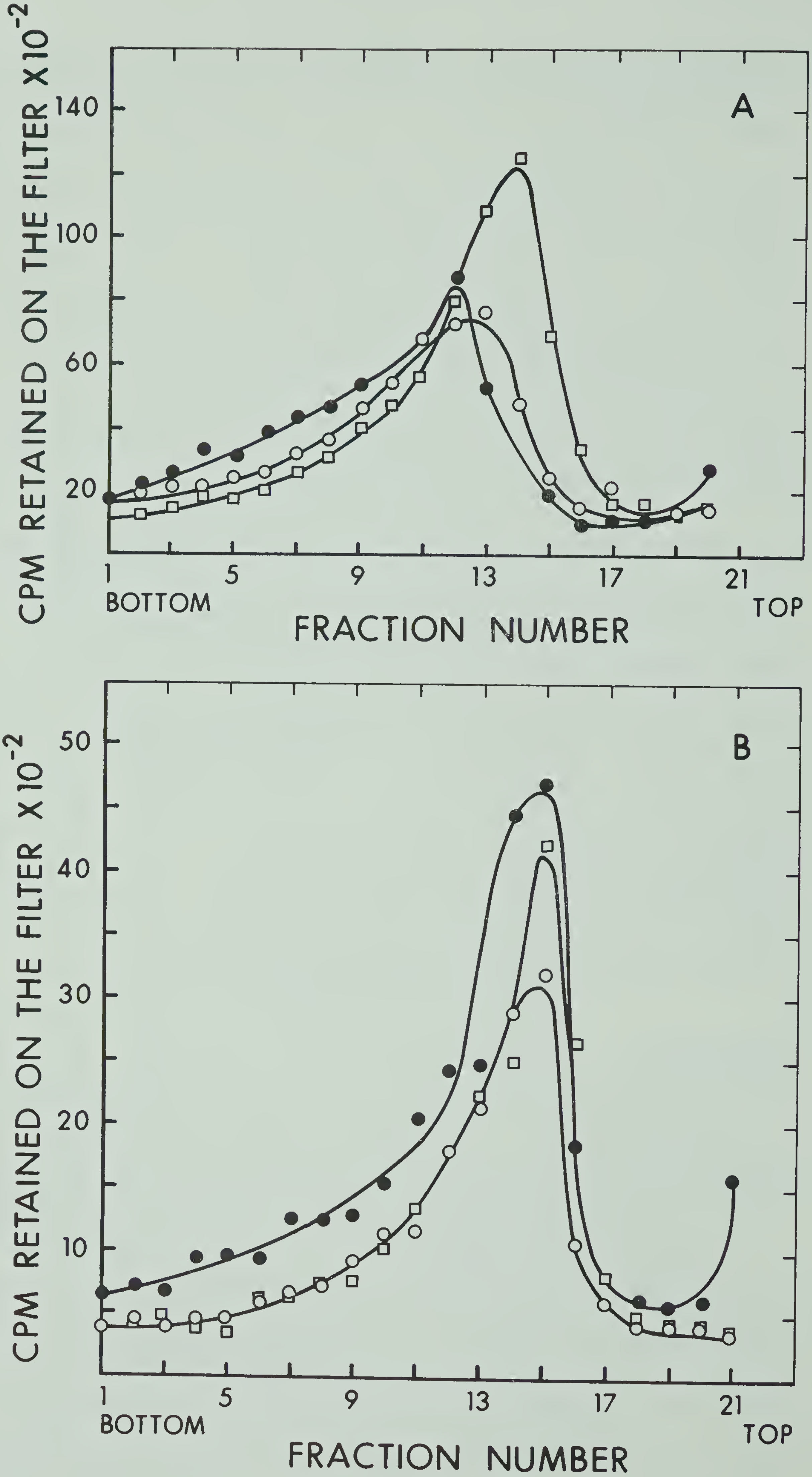

A
CPM RETAINED ON THE FILTER X10$^{-2}$
140
100
60
20
1
5
9
13
17
21
BOTTOM
TOP
FRACTION NUMBER
B
CPM RETAINED ON THE FILTER X10$^{-2}$
50
40
30
20
10
1
5
9
13
17
21
BOTTOM
TOP
FRACTION NUMBER

Figure 3.11. The effect of various mono- and divalent cation concentrations on the sedimentation characteristics of F-pili in sucrose gradients

Equivalent amounts of concentrated F-pili were layered onto linear gradients of 18 - 36% sucrose in the indicated salt solution. Gradients were centrifuged for 3 hours at 35,000 rpm. Fractions of 12 drops each were collected by puncturing the bottom of the centrifuge tubes. To each fraction was added 1.2 ml. 1.0 M NaCl and approximately 20,000 cpm of $P^{32}$-labelled R17. After a 12-minute incubation at 37°, the contents of each tube were pipetted onto a GA-6 filter. Each filter was washed with three 5 ml. aliquots of 1.0 M NaCl, then dried. Filters were assayed for radioactivity in a liquid scintillation counter. Different pili preparations were used in A and B. Figure A: Buffer D (●); 25 mM $MgCl_2$ in Buffer D (o); 100 mM $MgCl_2$ in Buffer D (□). Figure B: Buffer D (●); 25 mM NaCl in Buffer D (o); 0.45 M NaCl in Buffer D (□).

carried out using this new filter showed that preformed phage-pili complexes dissociate when exposed to a solution of a lower salt concentration than was originally present during their formation.

Heeding the above precautions, a standard method for the preparation and assay of F-pili was devised, and it was found that F-pili could be prepared in a quantity sufficient for several hundred assays, and could be stored, undiluted, in an ice bath with negligible loss of phage-adsorbing activity over a six-day period.

When experiments were performed under the conditions of minimal background radioactivity, the following observations were made. In 0.01 M Tris (pH 7.3) (Buffer D), adsorption of phage to F-pili was very low, reaching only 5% or less of the maximal level obtained under optimal conditions. This was of importance since all salt solutions were prepared in this buffer, and the lowest possible base line of adsorption was desired.

The addition of increasing amounts of monovalent cations (all as chlorides) caused a progressive increase in the amount of phage adsorption. Sodium, potassium, and ammonium ions were effective in promoting phage attachment in the order $Na^+ > K^+ > NH_4^+$.

The slopes of the curves relating attachment to the molarity of the salt solutions are less steep with monovalent than with divalent ions. Although divalent cations, in general, displayed their optimal stimulation at lower concentrations, the level of maximal attachment was not significantly higher than that eventually reached using monovalent cations. The steepest curve was obtained with $Ni^{++}$, although the highest level reached with this ion was lower than with other divalent ions.

No reliable information could be obtained about trivalent ions because of precipitation which occurred in a number of the incubation mixtures. This precipitate was able to effectively block passage of free phage through the filter.

When the data for mono- and divalent cations were replotted as a function of ionic strength, it was found that the stimulation of phage adsorption to F-pili was a function of the ionic strength, and independent of the valency of the cations.

It is now possible to resolve the apparent paradox that phage attachment to cell-free F-pili "requires" divalent cations (Valentine and Strand, 1965), while attachment to cell-bound F-pili has no such requirement (Paranchych, 1966). In the former case, the experiments had been done in 50 mM

Tris buffer, a medium in which any salt, mono- or divalent, would have shown a stimulation of adsorption. In the case of phage adsorption to cell-bound F-pili, these experiments were carried out in a complete minimal salts medium (without magnesium ion) in which the salt concentration was approximately 0.25 M (contributed mainly by the chlorides of $Na^+$, $K^+$, and $NH_4^+$). Under these conditions, one would not see any appreciable effect on adsorption resulting from the addition of small amounts of divalent ions.

Although it has been possible to describe the action of various cations, and thus, to clarify experimental differences described above, a major problem still faces investigators, namely: what is the mechanism of action of cations *vis à vis* bacteriophage adsorption to F-pili? In the case of RNA phage attachment to male-specific pili of *E. coli*, the data are not consistent with the charge neutralization hypothesis of Puck *et al*. (1950), although it seems likely that surface charge does play an important role in phage-cell interaction. Perhaps this question will be resolved when more information is available concerning the chemical nature of F-pili and the charge localization on the bacteriophage, and more particularly, on the A protein.

# IV. THE PHYSIOLOGICAL ASPECTS OF F-PILI GROWTH

## Introduction

Considering the importance of F-pili in RNA phage infection, it is surprising that so little is known about their growth.

Electron micrographs of growing aerated cultures of male bacterial cells have revealed a decrease in the number of cell-associated F-pili after overnight incubation of the culture (Brinton, 1965). This, as well as various other observations, has led Brinton to propose that F-pili development may involve a continuous growing out from the cell, with subsequent detachment of the pili when they achieve a certain length. This might be thought of as the "continuous growth" hypothesis. An alternative situation could exist, however, which would involve growth of pili to a certain size, after which pili growth would cease and the pili would remain attached to the bacteria. This might be thought of as the "limited growth" hypothesis.

The latter hypothesis would predict that one should find little or no accumulation of cell-free pili in a growing culture, while cell-associated pili should increase in proportion to cell growth. If the former hypothesis

were correct, on the other hand, one should find a substantial accumulation of cell-free pili in a growing culture.

It should thus be feasible to distinguish between these two hypotheses simply by measuring the relative amounts of free- and cell-associated pili in a growing culture at various cell densities. Such measurements should ideally be carried out with the aid of an electron microscope. Unfortunately, an electron microscope was available to us only on a limited basis through the courtesy of Dr. T. Yamamoto of the Department of Microbiology. We thus turned to an alternative method of measuring cell-free and cell-associated pili; namely, the filtration technique described in the previous chapter. Although the procedure was initially designed to study the attachment of phage to pili, it seemed likely that the technique could be adapted for use in measuring the concentration of pili, providing one could be certain that the bulk of the pili are retained by the filter.

Preliminary experiments showed that this was indeed possible, and indicated further that the normal pili growth cycle probably involves little, if any, detachment of pili from bacteria. We were thus encouraged to pursue the problem further, and the studies which were carried out are described in what follows.

## Methods and Materials

It has been pointed out by Brinton, and observed in our own laboratory, that a portion of many F-pili preparations consists of fragments small enough to pass through the 0.45 μ pores of the GA-6 filter. It is therefore important for our assay procedure to establish culture conditions under which all pili in a culture remain intact. To illustrate the effect that shearing has upon the size of F-pili, Brinton and Beer (1967) homogenized a concentrated pili preparation for various intervals in a Servall Omni-mixer (at a rheostat setting of 60), and determined the amount of F-pili retained on a filter by using attached labelled phage as a marker. The results showed that F-pili were completely disrupted into filterable fragments by 20 minutes of blending. Although this violent agitation clearly breaks up F-pili, it would be quite reasonable to suspect that cultures, in which shearing forces were at an absolute minimum, would contain mostly long F-pili. This is generally supported by electron microscopic observations, but it must be stressed again that it has been impossible to carry out extensive quantitative studies of this kind.

The low shear conditions employed in this study consisted of swirling cells in large flasks (to maximize the surface area of the culture) at a speed of 90 rpm in a rotary

water bath shaker. At this speed, the medium was agitated sufficiently to keep the cells well aerated, but no frothing or bubbling of the medium was observed. That these conditions provided adequate aeration of the culture was shown by the fact that the growth rate of the bacteria was identical to that of more vigorously aerated cultures.

The examination of cell-associated and cell-free F-pili necessitates the filtration of whole cells. It was found that filtration of cells at a density of 1 x $10^8$ bacteria per ml. occurs at a much slower rate than the filtration of free pili, suggesting that bacterial cells can block the pores of the filter. If such were the case, it is possible that phage could be trapped on the filter even though they were not part of a phage-pili complex. An experiment was thus carried out to determine whether the passage of phage through the filter can, in fact, be obstructed by cells. E. coli K12/R17, a phage resistant (non-adsorbing) mutant of E. coli K12, was therefore isolated and used for this experiment. Two incubation mixtures were prepared which contained equal amounts of free F-pili in 9 ml. of glucose TMM at 37°. To one of the incubation mixtures was added a total of 9 x $10^8$ cells of E. coli K12/R17. Equal amounts of $P^{32}$-labelled phage were added to each incubation mixture, and samples were removed at 1, 3, 6, and 12 minutes

after phage addition and filtered in the usual manner. The filters were then washed and dried, and the radioactivity of the dried filters was plotted versus time, as shown in Figure 4.1. It may be seen that the same curve adequately describes both sets of data, indicating that non-specific trapping of free phage by bacterial cells (lacking F-pili) does not occur. In subsequent experiments, cultures which were assayed for F-pili by filtration were always first diluted to a constant cell density of 1 x $10^8$ cells/ml. This step in the procedure insured a constant filtration rate for all the samples assayed, and eliminated the necessity of having to increase the amount of radioactive phage in the incubation medium as the cell density became greater.

### The effect of various shearing forces on the proportion of free and connected F-pili in a culture of E. coli K12

It was evident from the outset of these studies that, to obtain an unequivocal answer to the question, it would be necessary to know the extent to which free pili in a growing culture were produced by mechanical forces imposed during the growth and handling of the culture. One might expect that, in studies of this nature, pili could be broken off from cells in two ways: (1) as a result of shear forces imposed when samples are pipetted or passed through a hypodermic needle, and (2) as a result of shear forces imposed on the culture by normal aeration procedures.

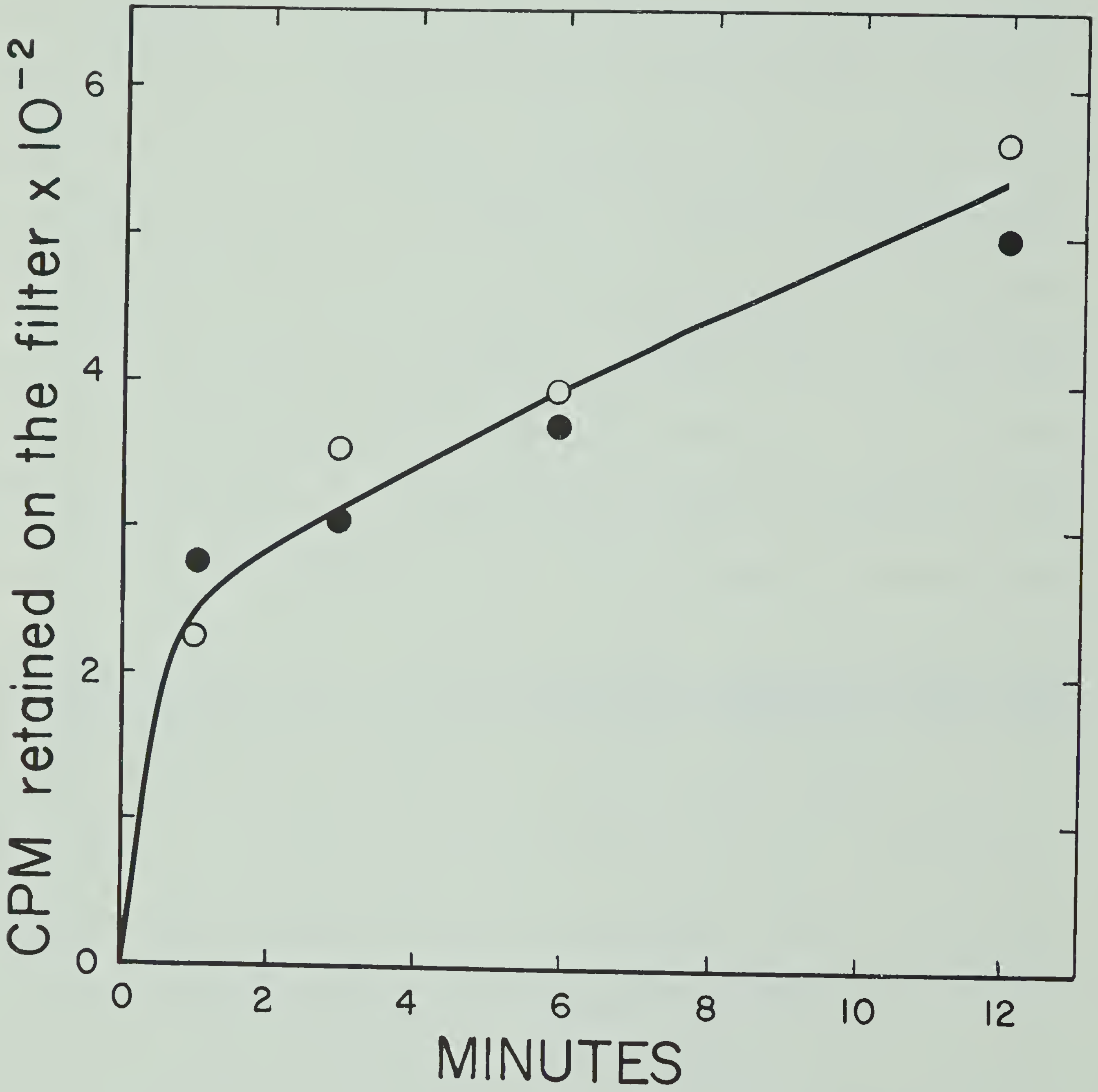
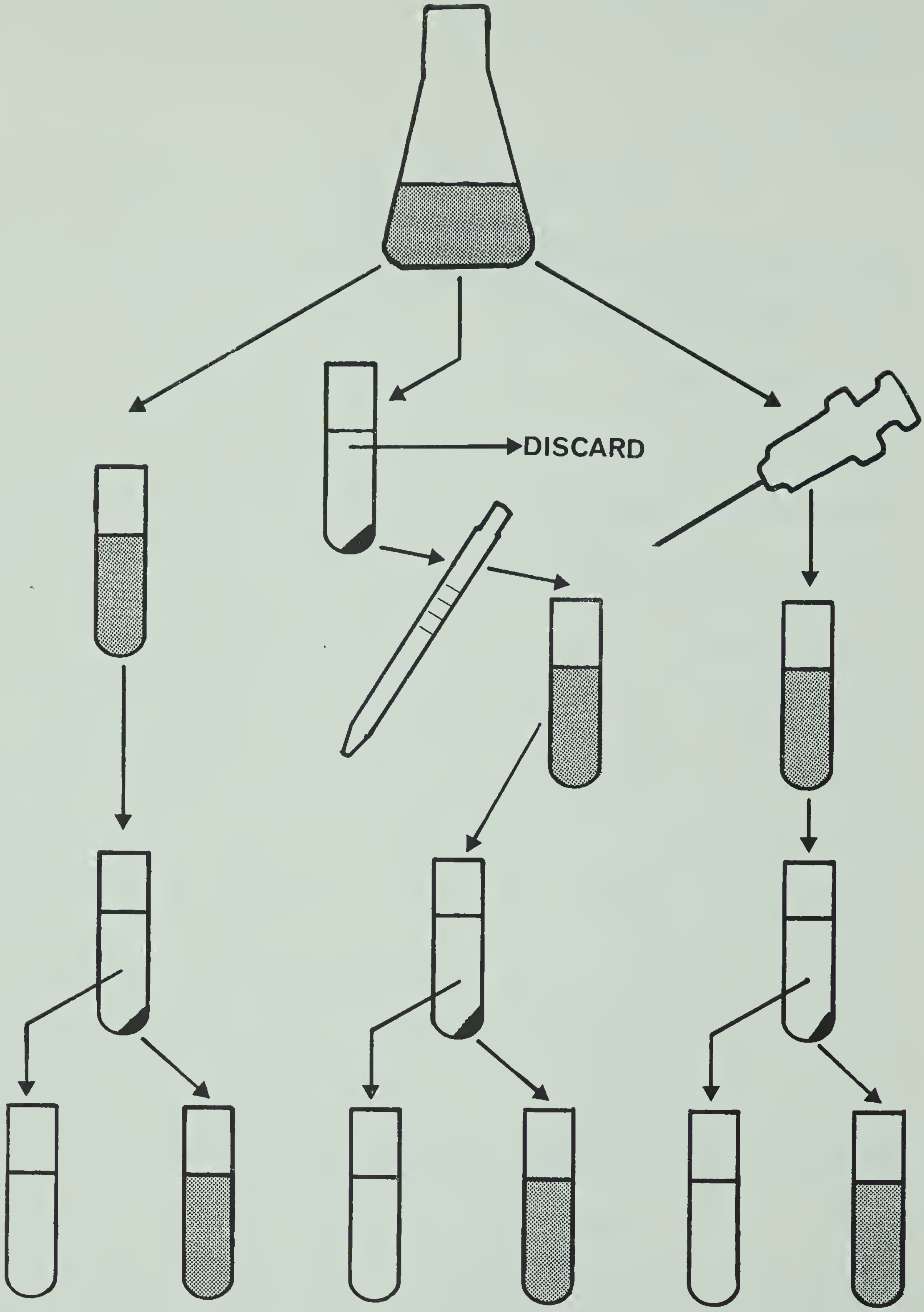

CPM retained on the filter x 10$^{-2}$
0
2
4
6
0
2
4
6
8
10
12
MINUTES

*Figure 4.1*. The effect of phage resistant (non-adsorbing) *E. coli* cells in the standard filtration assay of free F-pili

Two incubation mixtures were prepared which contained equal amounts of free F-pili in 9 ml. of complete medium at $37^o$. To one of the incubation mixtures was added a total of $9 \times 10^8$ cells of *E. coli* K12/R17, a phage resistant (non-adsorbing) mutant of *E. coli* K12. Equal amounts of $P^{32}$-labelled phage R17 were added to each incubation mixture, after which samples were removed at 1, 3, 6, and 12 minutes and filtered according to normal procedures. F-pili (●); F-pili + K12/R17 (o).

Experiments were thus carried out to examine the effect of these treatments on the formation of cell-free pili as follows:

(a) The effect of pipetting, or passing of the culture through a narrow bore needle on the state of F-pili in a bacterial culture. A culture of *E. coli* K12 was grown with slow shaking in glucose TMM to a density of 1 x $10^8$ cells per ml. The culture was chilled and divided into three 20 ml. aliquots. One portion was centrifuged 5 minutes at 10,000 g. to sediment the bacteria. The supernatant was discarded and the pellet was resuspended with repeated pipetting to its original volume in culture medium. A second 20 ml. aliquot was passed once through a #18 needle. The third 20 ml. fraction, which served as the control (unsheared) culture, was gently poured into a centrifuge tube. All three cultures were subdivided by centrifugation into a supernatant and a pellet, which was gently resuspended to its original volume in culture medium. Resuspension was accomplished by gentle swirling with a small amount of medium in the centrifuge tube. This entire procedure is outlined in the flow sheet of Figure 4.2. Equal amounts of $P^{32}$-labelled phage were added to 9 ml. of each of the six pili or cell samples. To avoid regeneration of the pili, all incubations were performed at $0^{\circ}$. Two ml. aliquots of



graph symbols ● ○ x

Figure 4.2. Schematic diagram of the subdivision of a bacterial culture into fractions for the assay of free and connected F-pili after various shearing treatments

Details of this experiment are described in the text.

each of these incubation mixtures were filtered at various intervals over a period of 12 minutes; after which the filters were washed, dried, and the radioactivity thereon measured in the usual manner. The filtration results, shown in Figures 4.3 (cells) and 4.4 (pili), indicate that the normal level of free pili in cultures grown under low shear conditions is quite low. In addition, it was found that either pipetting or sampling by means of a syringe caused considerable depiliation of the bacterial cells. It appears that either procedure removes F-pili to a similar extent, but that resuspension of the cells causes greater fragmentation of the pili than does a single passage through the hypodermic needle. This latter conclusion is based on the observation that the supernatant from the sample that had been pipetted apparently contained fewer pili than did the supernatant from the sample passed through a syringe. If nearly equivalent amounts of F-pili were removed from the cells, as the data shown in Figure 4.3 suggest, then there should be equal amounts of free pili in the two supernatants, unless in one case the shearing forces were sufficient to reduce a large proportion of the F-pili to filterable fragments. These results, therefore, show quite conclusively that F-pili are sensitive to shearing forces.

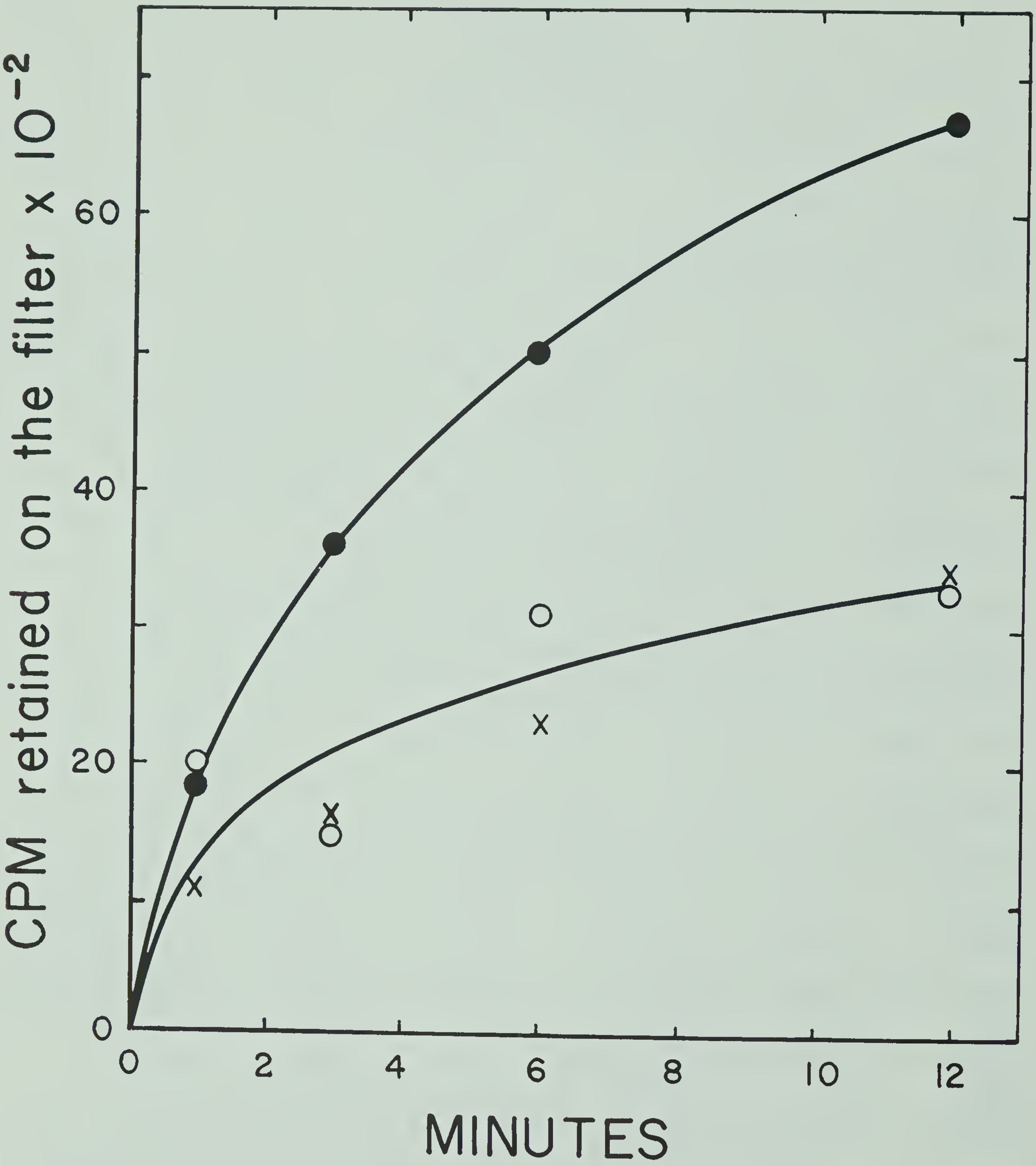

CPM retained on the filter x $10^{-2}$
0
20
40
60
0
2
4
6
8
10
12
MINUTES

Figure 4.3. The effect of various shearing forces on the amount of cell-associated F-pili in a culture of *E. coli* K12

A culture of *E. coli* K12 was grown with slow shaking in glucose TMM to a density of 1 x $10^8$ cells per ml., after which the culture was chilled and divided into three 20 ml. aliquots. One portion was centrifuged at 10,000 g. to sediment bacteria. The pellet was resuspended to its original volume in fresh culture medium. A second 20 ml. aliquot was passed through an 18-gauge needle, and the third 20 ml. fraction was left untreated. Each culture was further subdivided by centrifugation into a supernatant and a pellet (which was gently resuspended to its original volume in fresh culture medium). Equal amounts of $P^{32}$-labelled phage were added to each resuspended cell sample (chilled to $0^o$ to prevent pili regrowth) and 2 ml. aliquots were filtered and their radioactivity counted according to standard procedures. Control (unsheared) culture (●); culture passed through an 18-gauge needle (o); cells sedimented and resuspended with normal pipetting (x).

See accompanying schematic diagram (Figure 4.2).

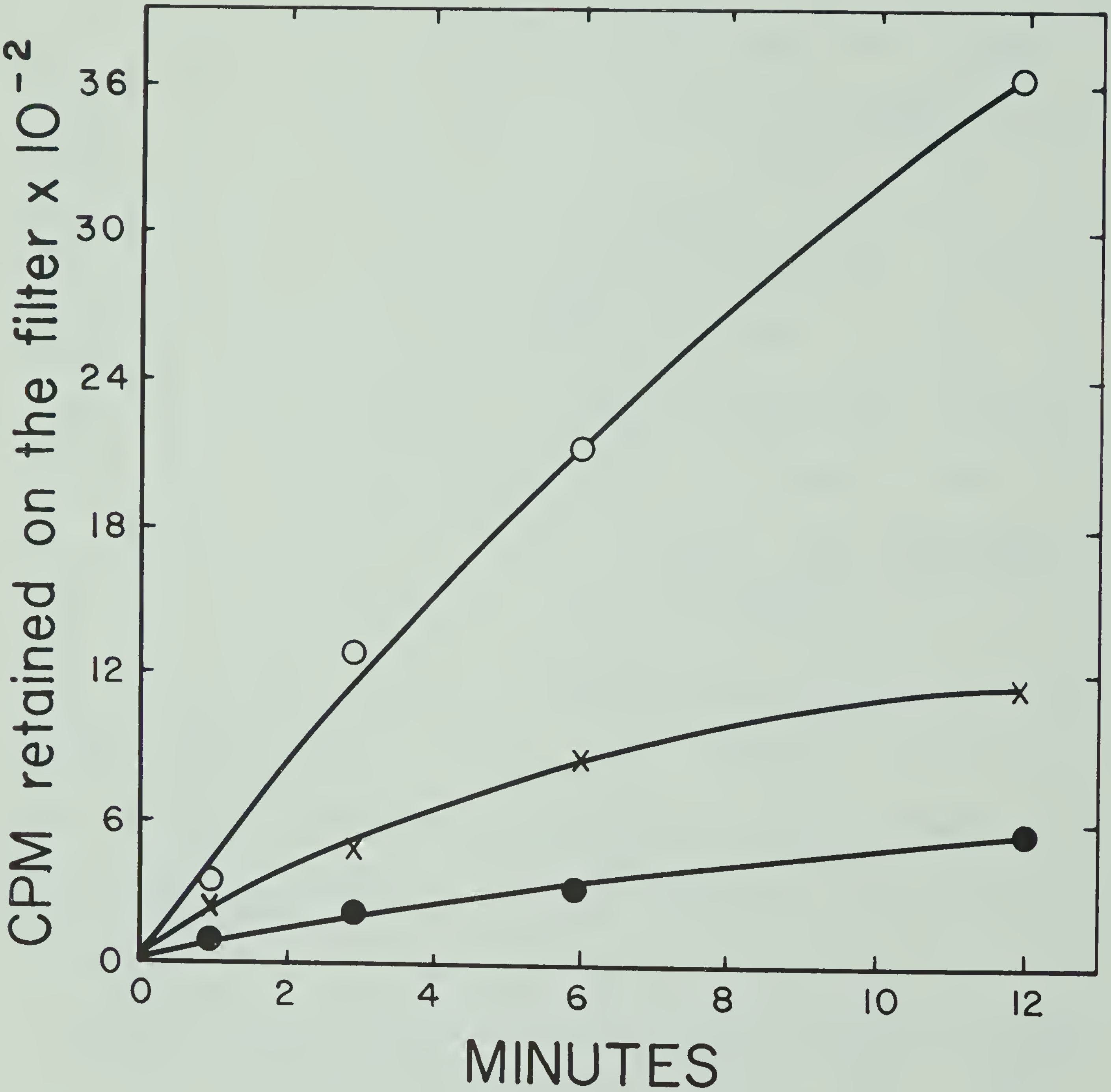

CPM retained on the filter x 10⁻²
0
6
12
18
24
30
36
0
2
4
6
8
10
12
MINUTES

Figure 4.4. The effect of various shearing forces on the amount of cell-free F-pili in a culture of *E. coli* K12

The experimental details for this figure are described in detail in Figure 4.3. Whereas the previous figure shows data for the resuspended cells, these are the data for the filtration assay of the cell supernatants. Incubation was at 0°. F-pili from the control (unsheared) culture (●); F-pili from the culture which had been passed through an 18-gauge needle (o); F-pili from the cells which had been sedimented and resuspended using normal pipetting (x). See accompanying schematic diagram (Figure 4.2).

(b) The effect of aeration on the state of F-pili in a growing culture. A normal means of providing oxygen to a culture is by forced aeration through a sparger. Passage of air under pressure through a culture causes considerable turbulence, and it seems possible that F-pili may be removed from the cells under these conditions. A simple experiment was therefore performed to test this hypothesis. A culture of *E. coli* K12 was grown to mid-log phase in glucose TMM under conditions of slow shaking. A sample was then removed, chilled, and assayed for free and connected F-pili as previously described, after which the culture was vigorously aerated. Additional samples were removed from the culture after 5 minutes and again after 15 minutes. These cultures were also chilled, diluted to 1 x $10^8$ cells per ml., and assayed for free and connected F-pili. The result of this experiment is illustrated in Figure 4.5. The amount of connected pili decreased by 75% during the first 5 minutes of aeration, and by 15 minutes, the culture contained less than 5% of the original level of detectable cell-associated pili. Aeration-sheared pili appeared in the supernatant of the culture at 5 minutes, then their level subsequently decreased, presumably due to degradation of F-pili into filterable fragments. It should be noted that the aeration used in this experiment was considerably more vigorous than

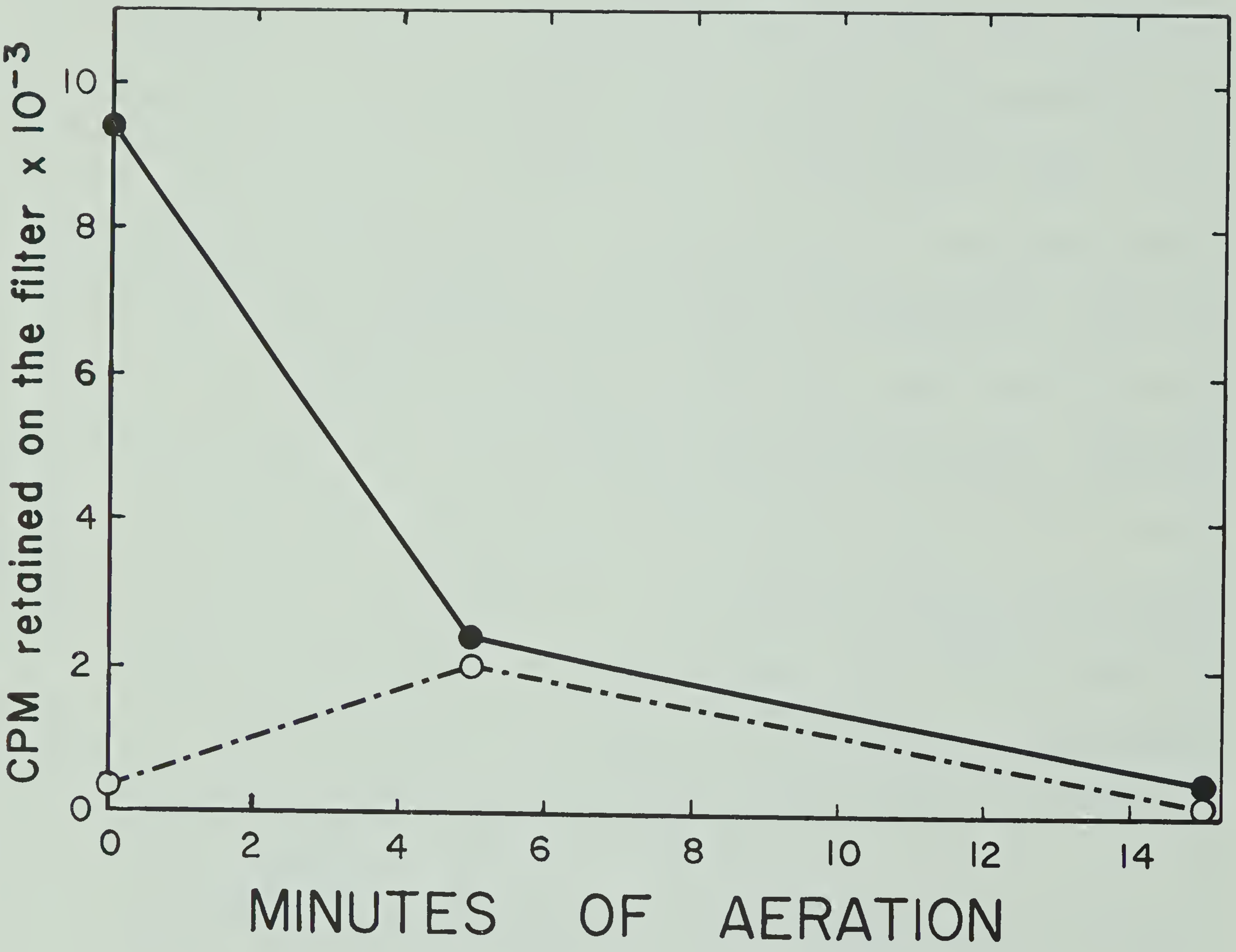

CPM retained on the filter x $10^{-3}$
0
2
4
6
8
10
0
2
4
6
8
10
12
14
MINUTES OF AERATION

Figure 4.5. The effect of aeration on the proportion of free and connected F-pili in a growing culture of *E. coli* K12

A culture of *E. coli* K12 was grown to 2 - 3 x $10^8$ cells per ml. in glucose TMM under conditions of slow shaking. A sample was removed, chilled, diluted to 1 x $10^8$ cells per ml., and assayed for free and connected F-pili as previously described. The culture was then vigorously aerated and additional samples were removed after 5 minutes and again after 15 minutes. These samples were also chilled, diluted to 1 x $10^8$ cells per ml., and assayed for free and connected F-pili. Cell-associated F-pili (—●—); free F-pili (--o--).

that used normally in oxygenating cultures. Nevertheless, this experiment points out the fact that turbulence in a culture can also depiliate cells. (A similar type of violent agitation is encountered in continuous flow centrifugation, with the result that F-pili are normally found in the culture supernatant rather than attached to the cells in the bacterial pellet.)

## An experiment to test the "continuous growth" and "limited growth" hypotheses of F-pili elongation

The data presented in this chapter indicate that any culture or transfer procedure, except the most gentle, will cause some depiliation of bacterial cells. Overnight aeration could, therefore, have accounted in part for the decrease in cell-bound F-pili noted by Brinton (1965). Nevertheless, it was felt that the physiology of pili growth could be tested in the simple experiment which was outlined at the beginning of this chapter; namely, measurement of the relative amounts of cell-free and cell-associated F-pili in growing cultures of *E. coli* K12. The experiment is based on the premise that if F-pili are normally released from growing cells ("continuous growth" hypothesis), they will accumulate in the culture medium, whereas, if they grow according to the "limited growth" scheme, the concentration of cell-free pili in the medium of a growing culture would remain very low.

The actual experiment was carried out as follows. A slowly shaking culture of E. coli K12 was grown in glucose TMM to a cell density of 2 x $10^8$ bacteria per ml. at which time an aliquot of the culture was removed and chilled. Growth of the culture was continued, and additional aliquots were removed at cell densities corresponding to 4 x $10^8$ and 7 x $10^8$ cells per ml. All cultures were chilled and diluted with culture medium to a cell density of 1 x $10^8$ cells per ml. One-half of each diluted culture was centrifuged to sediment bacterial cells, and the supernatant, containing the free F-pili was saved. The samples were then assayed for cell-free pili (supernatant solution remaining after removal of cells by centrifugation), and cell-associated pili (whole culture assayed for pili and then values corrected to account for the amount of cell-free pili). The values (cpm retained on filters) obtained after a 40-minute incubation period were multiplied by 2.4 or 7 to account for the dilution of the original samples, and the values so obtained were plotted against the cell densities in the original samples (Figure 4.6). It may be seen that the amount of free F-pili which accumulated in the culture during a period of almost two generations was approximately one-tenth of the amount of cell-associated pili produced by the culture during this same period.

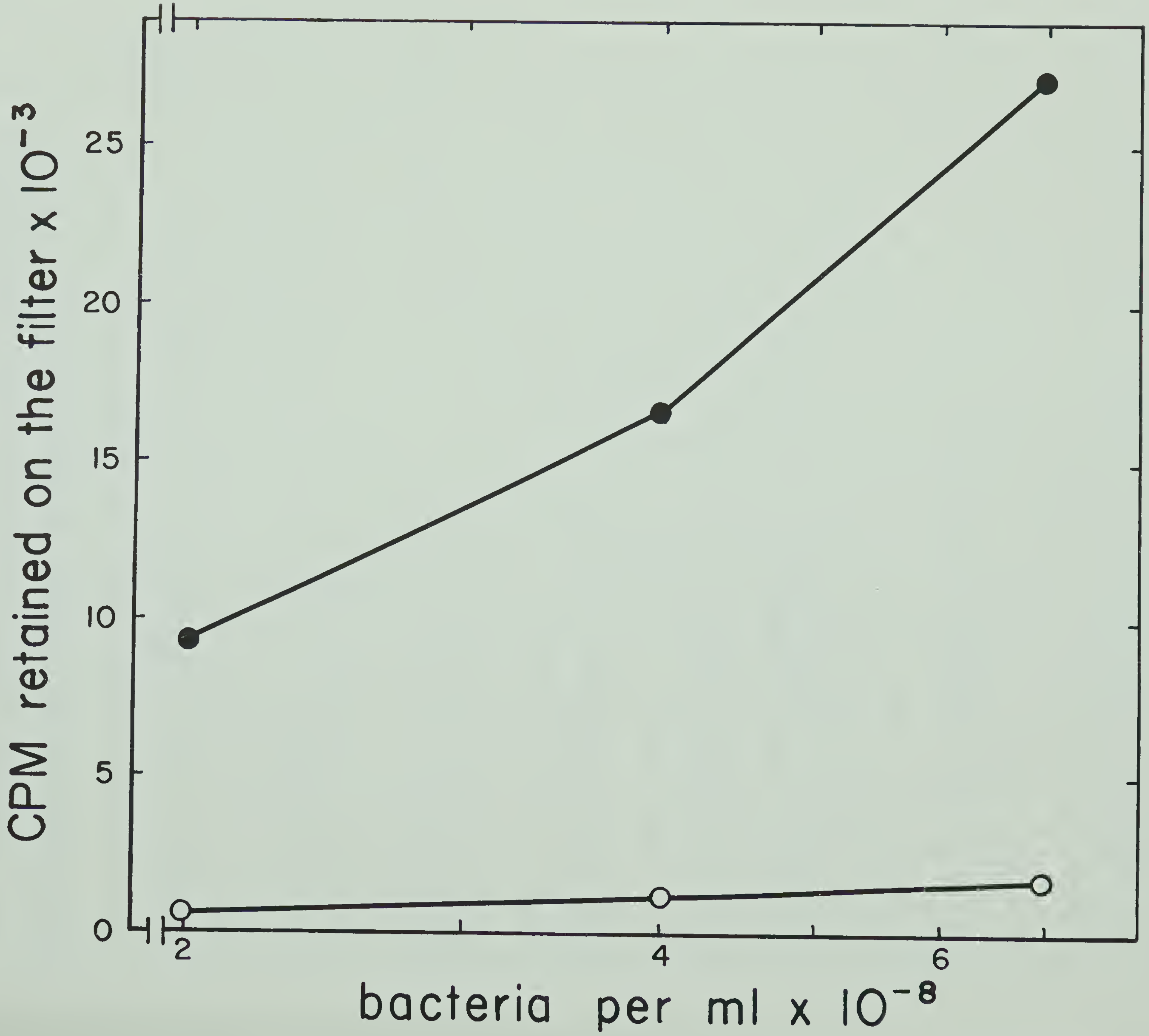

CPM retained on the filter x $10^{-3}$
25
20
15
10
5
0
2
4
6
bacteria per ml x $10^{-8}$

Figure 4.6. Amounts of total and cell-free F-pili in a growing culture of *E. coli* K12

A slowly shaking culture of *E. coli* K12 was grown in glucose TMM to a cell density of $2 \times 10^8$ cells per ml., at which time an aliquot of the culture was removed and chilled. Growth of the culture was continued and additional aliquots were removed at cell densities corresponding to $4 \times 10^8$ and $7 \times 10^8$ bacteria per ml. All cultures were diluted with culture medium to $1 \times 10^8$ cells per ml. for ease of filtration. One-half of each diluted culture was centrifuged to sediment bacterial cells, and the supernatant, containing the free F-pili was saved. The whole cultures and the pili supernatants from the diluted cells at each density were assayed by the normal filtration procedure with incubation at 0°. The maximal value of each timed adsorption curve (expressed as cpm retained on the filter) was multiplied by its dilution factor and these results are plotted versus original cell density. Cell-associated pili (●); cell-free pili (o).

It seems likely, therefore, that normal growth of F-pili does not involve a continuous growth and release mechanism, but that the growth of this organelle ceases when the pilus reaches a certain length, the pilus then remaining attached to the cell.

## Reversibility of phage adsorption to cell-free F-pili

The exceptional care which was taken to avoid shear forces in the previous experiments represents quite a departure from the normal culture conditions where cells are usually aerated or shaken rapidly. Under these latter conditions there would be free F-pili in the culture medium, and were such a culture to be infected with phage, these free pili could have a significant effect on the efficiency of infection, since they can bind large numbers of phage particles. The magnitude of this effect would depend on the reversibility of the binding to free F-pili, and the infectivity of phage particles which have been bound and have dissociated. More specifically, it is possible to ask whether phage attachment is readily reversible under conditions optimal for their attachment to F-pili, since it will be shown in the next chapter that interaction of phage with free F-pili does not result in a loss of infectivity.

The experiment to determine the reversibility of phage attachment is relatively uncomplicated, and the

equilibrium expression is that used for standard chemical reactions.

$$\emptyset + \text{total sites} \rightleftharpoons \emptyset\text{-filled sites}$$

$$\text{therefore:}\quad K_{\text{equil.}} = \frac{\emptyset\text{-filled sites}}{(\emptyset)\ (\text{total sites})}$$

Since, by definition, an attachment site is a locus on the pilus to which a phage particle can bind, the number of available or filled sites can be expressed as cpm of phage which can or do attach.

Since the amount of input phage was known, and the filled sites were determined directly by filtration of the phage-pili incubation mixtures, the only unknown quantity was the total number of available sites. The latter was determined for the specific preparation (i.e. containing 11 μg./ml. of protein) in the following manner.

Increasing amounts of phage were added to a constant amount (11 μg./ml.) of F-pili in the standard filtration assay. The incubation buffer was 0.5 M NaCl in Buffer D, and 2 ml. aliquots were filtered at intervals over a period of 60 minutes. A series of adsorption curves were obtained (Figure 4.7), from which the plateau value of each curve was plotted versus phage input according to the Lineweaver-Burke system, (i.e. $\frac{1}{\text{input cpm}}$ versus $\frac{1}{\text{attached cpm}}$ ) (Figure 4.8). A straight line was obtained, the ordinate intercept

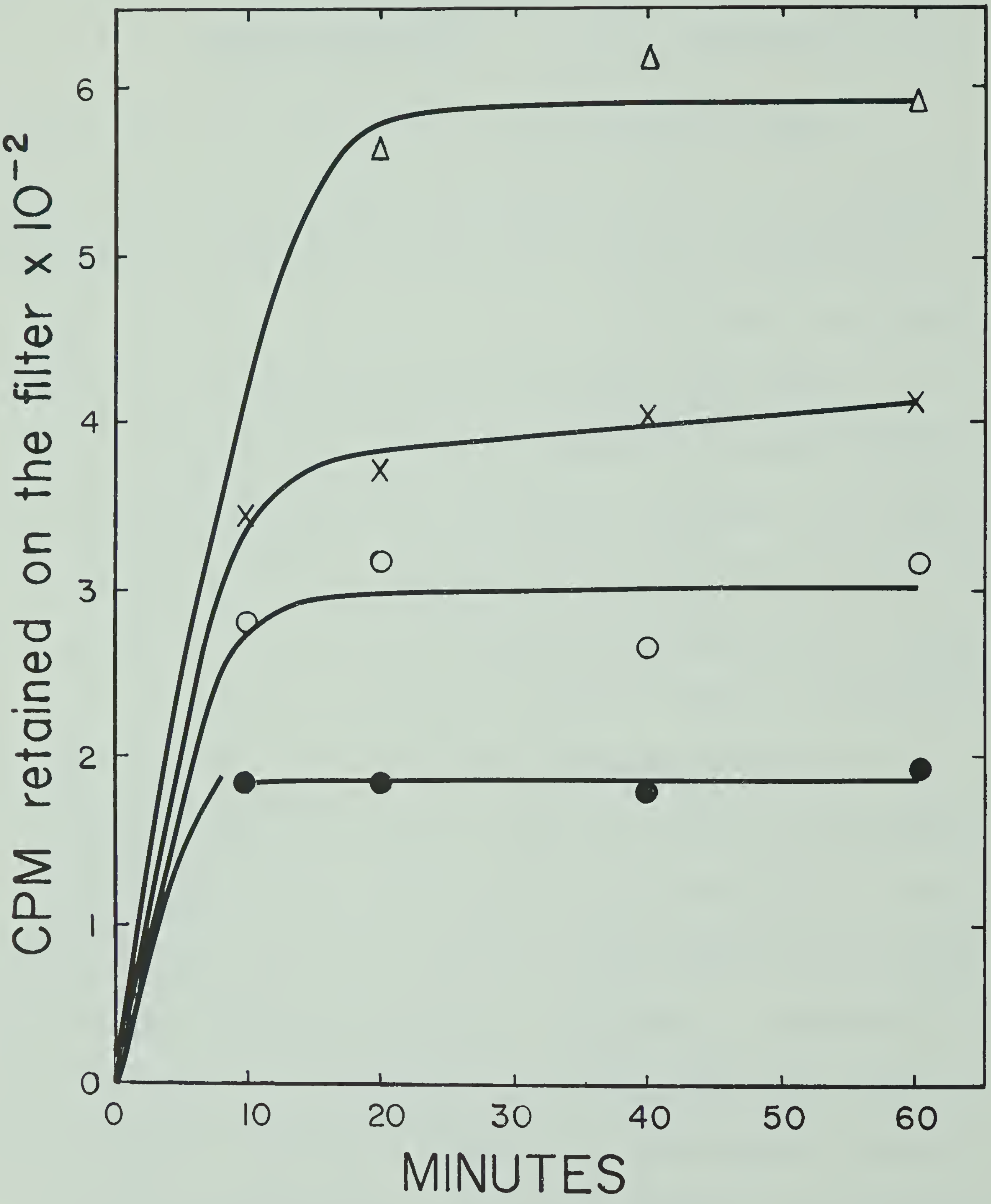

CPM retained on the filter x $10^{-2}$
0
1
2
3
4
5
6
0
10
20
30
40
50
60
MINUTES

Figure 4.7. The effect of increasing phage input on the filtration assay for free F-pili

Increasing amounts of $P^{32}$-labelled phage stock (6 x $10^8$ particles/ λ) were added to a constant amount of F-pili (11 μg./ml.) in 0.5 M NaCl (in Buffer D) at 37°. (Total volume = 9 ml.) Two ml. aliquots from the incubation mixtures at each phage concentration were filtered according to the standard procedure at the indicated times over a period of 60 minutes. 40 λ (●); 80 λ (o); 160 λ (x); 320 λ (△).

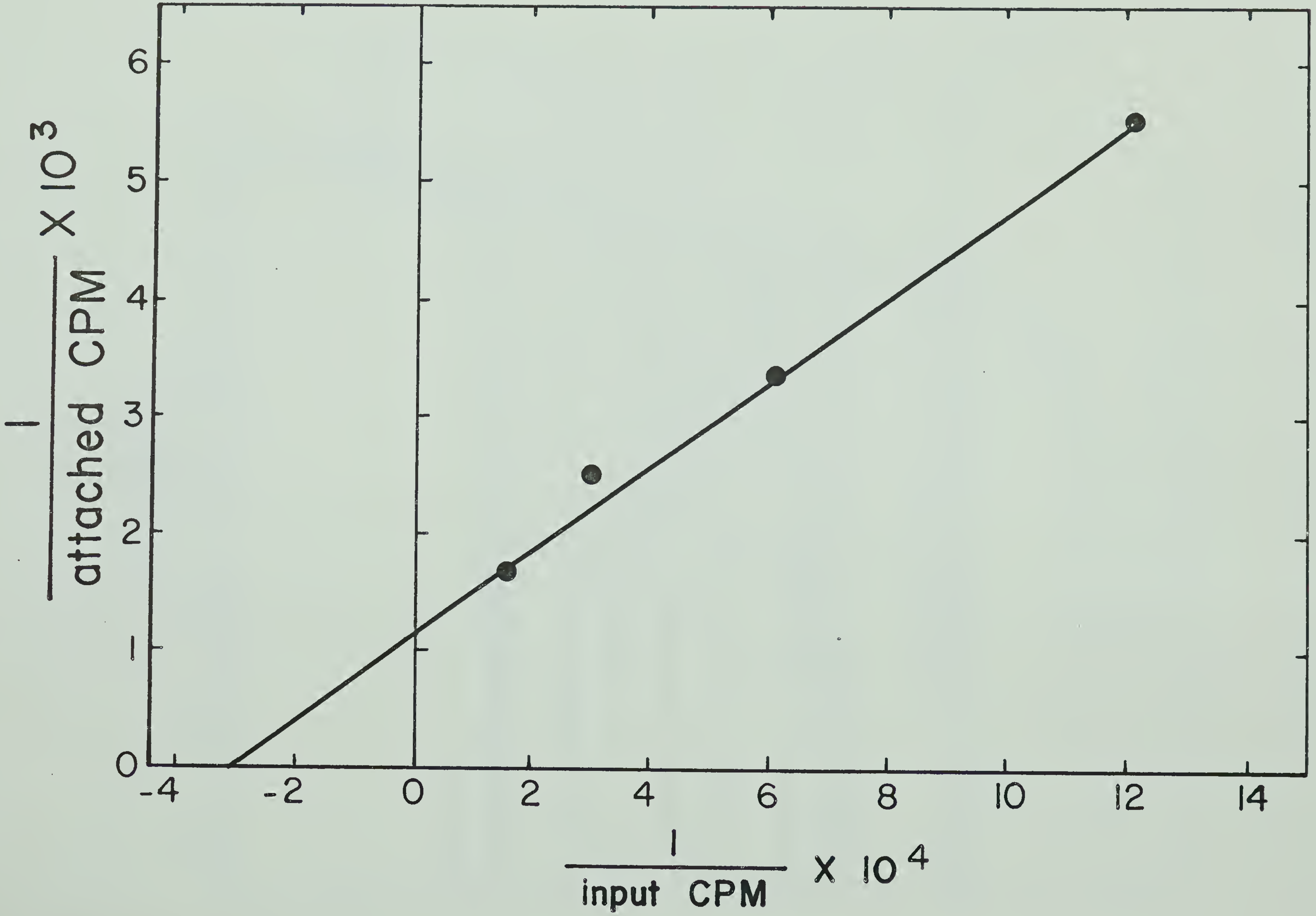

$\frac{1}{\text{attached CPM}} \times 10^3$
0
1
2
3
4
5
6
-4
-2
0
2
4
6
8
10
12
14
$\frac{1}{\text{input CPM}} \times 10^4$

Figure 4.8. The effect of increasing phage input on the attachment of phage to free F-pili

The 60-minute value of each adsorption curve of Figure 4.7 is plotted versus the input level of phage, according to the Lineweaver-Burke system (i.e. a double reciprocal plot).

(infinite phage input) of which represented complete saturation of pili in 2 ml. of solution. The total available sites were therefore calculated to be 894 cpm/2 ml. of pili solution (22 μg. protein), or $2.88 \times 10^{9}$ phage particles per ml.

Using the data from Figures 4.7 and 4.8, a numerical value of the equilibrium constant was calculated.

$$K_{\text{equil.}} = \frac{\text{Ø-filled sites}}{(\text{Ø})\ (\text{total sites})}$$

$$= \frac{300 \text{ cpm ml.}^{-1}}{(3300 \text{ cpm ml.}^{-1})\ (447 \text{ cpm ml.}^{-1})}$$

$$= 2.0 \times 10^{-4} \text{ ml./cpm}$$

Since the specific activity of the phage preparation was = $1.55 \times 10^{-7}$ cpm/particle:

$$K_{\text{equil.}} = 3.14 \times 10^{-11} \text{ ml./particle.}$$

These data indicate that phage are extremely weakly bound to free F-pili, confirming by the use of a different experimental procedure, the earlier findings of Brinton and Beer (1967).

## Discussion

The principal question posed in this chapter was whether F-pili remain firmly attached to, or grow out and spontaneously detach from the bacterial cells. An answer was obtained by measuring the relative amounts of free and cell-associated pili in a growing culture using a filtration assay.

Preliminary results suggested that F-pili are removed from cells much more easily than had been originally believed, and the following conditions of cell growth and handling were established to obtain cells which had been subjected to a minimum of shear. Small volumes of culture were incubated in large, gently swirling flasks. Large bore pipettes were used in transferring F-piliated bacterial cells, and the contents of the pipettes were gently drained rather than blown out. Furthermore, resuspension of cell pellets was carried out as gently as possible by light tapping of the centrifuge tube, rather than by mixing on a vortex mixer. The use of a syringe for removal of aliquots from a bacterial suspension was strictly avoided, except where piliation was of no concern.

Heeding these precautions, the pili release experiment was carried out. It was found that the amount of cell-associated pili that accumulated in a growing culture was approximately ten times greater than that of free F-pili. Subsequent to these investigations, it was reported (C. C. Brinton, Jr., personal communication) that free F-pili are able to readsorb to bacterial cells. If this is in fact the case, then further studies need to be carried out before it can be concluded with certainty whether pili growth proceeds via a "continuous growth" or "limited growth" process.

Reversibility of phage attachment to free F-pili was examined under optimal conditions of adsorption to determine how seriously the existence of free F-pili in a culture would lead to trapping of phage particles. (Phage can not infect bacteria while they are bound to free F-pili.) Binding of phage in 0.5 M NaCl (in Buffer D) was found to be extremely weak, and the equilibrium constant (3.14 x $10^{-11}$ ml./particle) was in good agreement with previous data of Brinton and Beer (1967). The difference between the values can be reasonably attributed to the amount of aggregation of the pili in the two preparations, and the fact that our value is expressed in terms of ml./particle rather than ml./PFU.

Although equilibrium favors the free phage, it is evident that when infecting cells which have been grown under conditions of vigorous aeration, many of the added phage particles will become attached to cell-free pili fragments and thus become unavailable for infection of the cell. This means that many previous efforts to describe the multiplicity of infection in terms of input PFU's per cell are probably not strictly correct.

# V. ENERGY REQUIREMENTS FOR THE UNCOATING OF BACTERIOPHAGE R17 RIBONUCLEIC ACID DURING THE EARLY STAGE OF INFECTION OF *ESCHERICHIA COLI* K12

## Introduction

The arbitrarily assigned stages of RNA phage infection, defined on the basis of their consistency with existing data, and by analogy with other viral infections are as follows: 1) the attachment of the phage to a specific receptor site on the host bacterium (this may be a two-stage attachment differentiated by the reversibility of the virus-cell association, e.g. reversible ⟶ irreversible attachment); 2) the phage nucleic acid release from the phage either by some extrusion mechanism or by a rupture of the capsid; 3) the penetration of the phage genome into bacterium and transportation to the site of replication, followed by the actual enzyme-catalyzed reproduction of the phage RNA and production of phage-specific proteins. According to this proposed scheme, if the F-pilus is the unique receptor organelle, free F-pili might be able to cause the uncoating of the bacteriophage nucleic acid and the released genome might be expected to be found within the pilus.

It was fortunate that a simple assay existed by which this hypothesis could be tested. The technique in question was the uncoating assay devised by Paranchych

(1966), based on the observation of Zinder (1963) that the RNA phage f2 does not give rise to plaques in the presence of pancreatic ribonuclease. It was reasoned that this observation could be explained according to the following scheme: subsequent to the attachment of the bacteriophage, the RNA is released from the capsid. At this time the nucleic acid is exposed to the culture medium. If ribonuclease is present, it hydrolyzes the phage RNA sufficiently to render it non-infectious. In the actual assay, a known number of PFU's of phage are incubated at 37° with the host, or with some portion of the host bacterium (the F-pilus), in a medium containing ribonuclease. During the incubation, aliquots are removed from the culture at various intervals and assayed for PFU's. A decrease in the number of PFU's in the culture occurs (Figure 5.1), indicating that the phage RNA is being uncoated and hydrolyzed (no loss of PFU's is observed if phage are incubated with ribonuclease in culture medium in the absence of cells). If no RNase is present in a bacterial culture at the time of infection, little or no net loss of infectivity occurs, since the loss of infectivity of each phage particle is accompanied by the formation of an infected cell. These cells, when plated on a lawn of male bacteria, behave as infectious centers, and if plated before lysis occurs within the culture they will give rise to

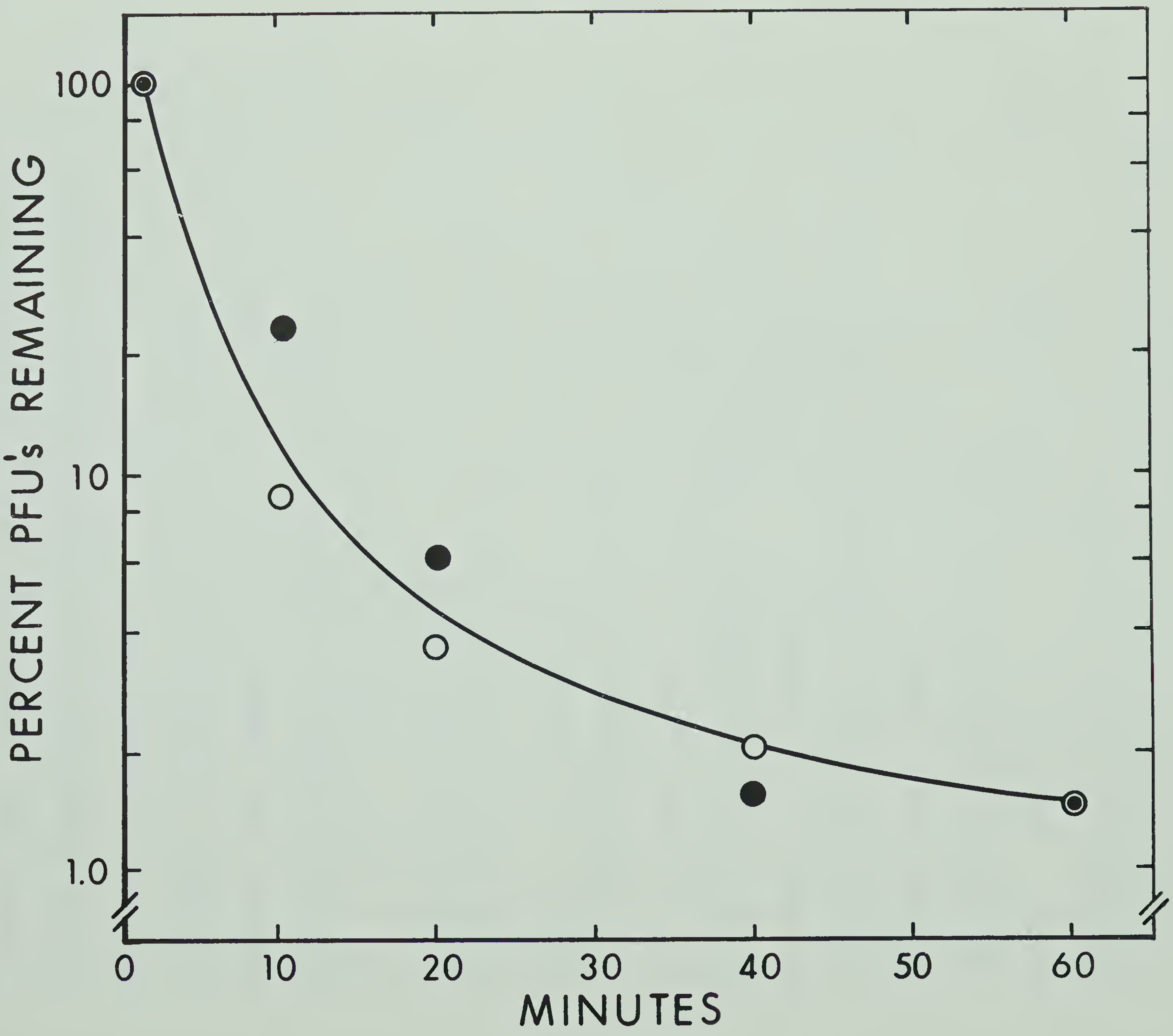

PERCENT PFU's REMAINING
100
10
1.0
0
10
20
30
40
50
60
MINUTES

Figure 5.1. Uncoating of R17 nucleic acid by cells of *E. coli* K12

Cells at a density of 2 x $10^8$ bacteria per ml. in glycerol or glucose-supplemented TMM, were infected in the presence of 100 µg./ml. Ribonuclease A at a multiplicity of 0.01 PFU/bacterium. Aliquots were removed at the indicated times for assay of residual infectivity. Glycerol TMM (o); glucose TMM (●).

single plaques. The addition of RNase to the incubation mixture thus prevents the formation of such infectious centers and allows the measurement of phage uncoating by following the loss of infectivity. It is to be noted that when phage is incubated under similar conditions with cell-free preparations of F-pili, no loss of infectivity is observed either in the presence or the absence of RNase.

Since previous data on the attachment of the phage pointed to the F-pilus as the receptor site, it was of interest to determine why these organelles, when present in a cell-free form, were non-functional beyond the level of simple phage adsorption. One obvious possibility was that the F-pilus is a part of a larger functional unit which must be intact to retain activity. The structural relationship of the F-pilus to the rest of the bacterium is still obscure. If, as has been proposed by Brinton (1967), the F-pilus is lipoprotein in nature, it could be a part of a membrane system which is inactive if disrupted. To study F-pili activity from this standpoint, it would have been necessary to isolate intact membranes from the bacterium, an area of research which is technologically complex, even in the light of the most recently reported separations of the cell surface layers of _E_. _coli_ (e.g. Miura, 1968).

In addition to the possibility that F-pili are part of a larger membrane structure that is rendered inactive if disrupted, Brinton (1965) has advanced a second possible explanation for the observation that free F-pili are unable to uncoat bacteriophage. It is commonly accepted that cells which are genotypically $F^+$, $F'$, Hfr, etc., have an extra piece of non-chromosomal DNA, the F-episome, which confers "maleness" on these cells. Brinton suggested that the locus of this DNA might be the base of the F-pilus, and that this DNA is somehow functional during nucleic acid transfer through the pilus. According to this premise, the cell-free F-pili would be incapable of uncoating phage, since this DNA is absent from these pili. Wendt (1967) attempted to determine whether or not pili contain DNA by studying the buoyant density of free F-pili in cesium chloride equilibrium density gradients, and comparing the values obtained with values known for the filamentous DNA coliphage, fl. The results were deemed inconclusive by the authors since the lipid content of F-pili was unknown. No reports of the chemical composition of F-pili are available to shed light on this question; thus, it would have been unrewarding to look for activity of F-pili as a function of a macromolecule that may or may not be present.

A third possible explanation for the inability of free F-pili to uncoat phage is that this step may require energy, and that the appropriate high energy compound is washed out during the isolation of the pili. Several indirect pieces of evidence provided some support for this hypothesis. Valentine and Wedel (1965) showed that viral RNA from phage f2 was unable to penetrate the bacterium at $0^{o}$. Knolle (1967) looked at the effect of temperature, KCN, and $NaN_3$ on "invasion" of E. coli 3300 by the RNA phage fr, and concluded from the inhibition caused by these agents, that invasion required the active participation of the host. It thus seemed possible that the inability of free F-pili to uncoat phage R17 could reflect an uncoupling from a normal energy supply. To examine this possibility, an experiment was carried out in which a number of nucleoside triphosphates were added singly, and in combination to incubation mixtures of F-pili, phage R17, and ribonuclease. Even in the presence of these high energy compounds, the pili were unable to uncoat the bacteriophage.

This disheartening result prompted us to formulate a single hypothesis containing the essential elements of the three that have been discussed. It is as follows: an F-pilus, to be active in causing the release of the genome from a bacteriophage, must be an integral part of the

[illegible]

[illegible]

bacterial cell. It requires the presence of F-specific DNA, and energy supplied by the normal metabolism of the cell. To test this premise, experiments were carried out on intact cells, rather than on isolated F-pili. In such a system, the questions of membrane integrity and requirement for DNA could be ignored, and the need for an energy supply examined directly. It was believed that a demonstration of a direct relationship between the level of ATP, the most common high energy source used by biological systems, and the ability of bacterial cells to cause uncoating of the phage RNA, would indicate whether an energy requirement were involved. It was further reasoned that even if ATP were not directly involved for the uncoating reaction, its level would still reflect the energy state of the cell, since, in the presence of normal amounts of ATP, the cell should be able to synthesize most of the other high energy derivatives which it requires. In cases where levels of ATP or its derivatives are significantly below normal, uncoating should be inhibited.

The experimental design employed, was to carry out uncoating assays using intact cells in which the ATP levels were altered to a known extent by the addition of various metabolic inhibitors, or by variation of the culture conditions. Uncoating assays were carried out at several temperatures, using a single culture, to determine a rough

[illegible]

[illegible]

activation energy for the uncoating process. This latter experiment indicated that uncoating required energy of the same order of magnitude as do many typical biological reactions. Furthermore, a direct correlation between inhibition of uncoating and decreased levels of ATP was found in cells which had been cultured in glycerol-supplemented medium. In glucose-supplemented cultures, such a strict relationship was not apparent. In the course of experiments designed to resolve these discrepancies, data was accumulated on the inhibition of uncoating caused by the uptake of glucose and its analogue, 2-deoxy-D-glucose (2DG), into glucose starved cells. These experiments seemed to suggest that the overall energy level of the bacterial cell may not be as important as the membrane-associated or surface-available energy of the cell.

## Methods and Materials

Bacteriophage R17 and its host, *E. coli* K12, have been described in detail in a previous chapter.

## Media

Glucose TMM has been described in Chapter II. In certain of the uncoating experiments a second culture medium, glycerol TMM, was used. This medium was identical to glucose TMM except that 1.0 ml. 50% glycerol was

substituted for 1.0 ml. of 50% glucose and the amino acid concentrate was omitted.

## Uncoating assay

It is believed that, subsequent to its attachment, the RNA phage releases its nucleic acid upon or into the F-pilus. At this time the RNA which is exposed to the medium finds its way into the bacterial cell by some unknown mechanism. If ribonuclease is present in the medium, the RNA is rendered non-infectious by this enzyme. It is the primary purpose of this chapter to demonstrate that the uncoating of the viral RNA is a process which is dependent upon energy supplied by the host's metabolism.

Since the uncoating assay forms the basis for all the experiments described in this section, it seems pertinent to discuss this assay in detail, and to provide experimental supporting evidence which demonstrates the physical significance of this event. This can best be shown by the following experiment. A culture of E. coli K12 was grown to a density of approximately $1 \times 10^9$ cells/ml. in glucose TMM. Chloramphenicol, at 100 µg./ml., was added to the culture to stop protein synthesis, and 15 minutes later the cells were chilled in ice. A portion of the culture, amounting to 30 ml., was blended in the 75 ml. cup of the Servall Omni-mixer for 1 minute, using a powerstat setting of 50. A 15 ml.

portion of the blended culture was centrifuged at 10,000 rpm for 5 minutes, a condition which results in sedimentation of whole bacteria but leaves the free F-pili suspended in the supernatant solution. There were, therefore, three preparations used for this experiment: intact cells, blended cells (blended culture not subjected to centrifugation), and free F-pili. Ten ml. of each of these preparations, which had all been made to contain 100 μg./ml. of ribonuclease, were incubated at 37° with an amount of $P^{32}$-labelled phage R17 equivalent to a multiplicity of one PFU per bacterium. At various time intervals over a period of 90 minutes, 1 ml. samples of each culture were removed and rapidly added to 2 ml. cold 7½% trichloroacetic acid (TCA). The samples were allowed to stand in ice for approximately one hour during which time a precipitate formed in each. The precipitates were collected by vacuum filtration on Gelman fibreglass filters and were washed twice with two 10 ml. portions of chilled 5% TCA - 0.9% PPi (the pyrophosphate was included in the wash solution to chase soluble phosphate compounds). Filters were placed in scintillation vials and counted in a liquid scintillation counter using 10 ml. Bray's scintillation fluid (Bray, 1960). Figure 5.2 shows the results of this experiment. The data are plotted as the per cent of the input phage radioactivity which remains TCA insoluble

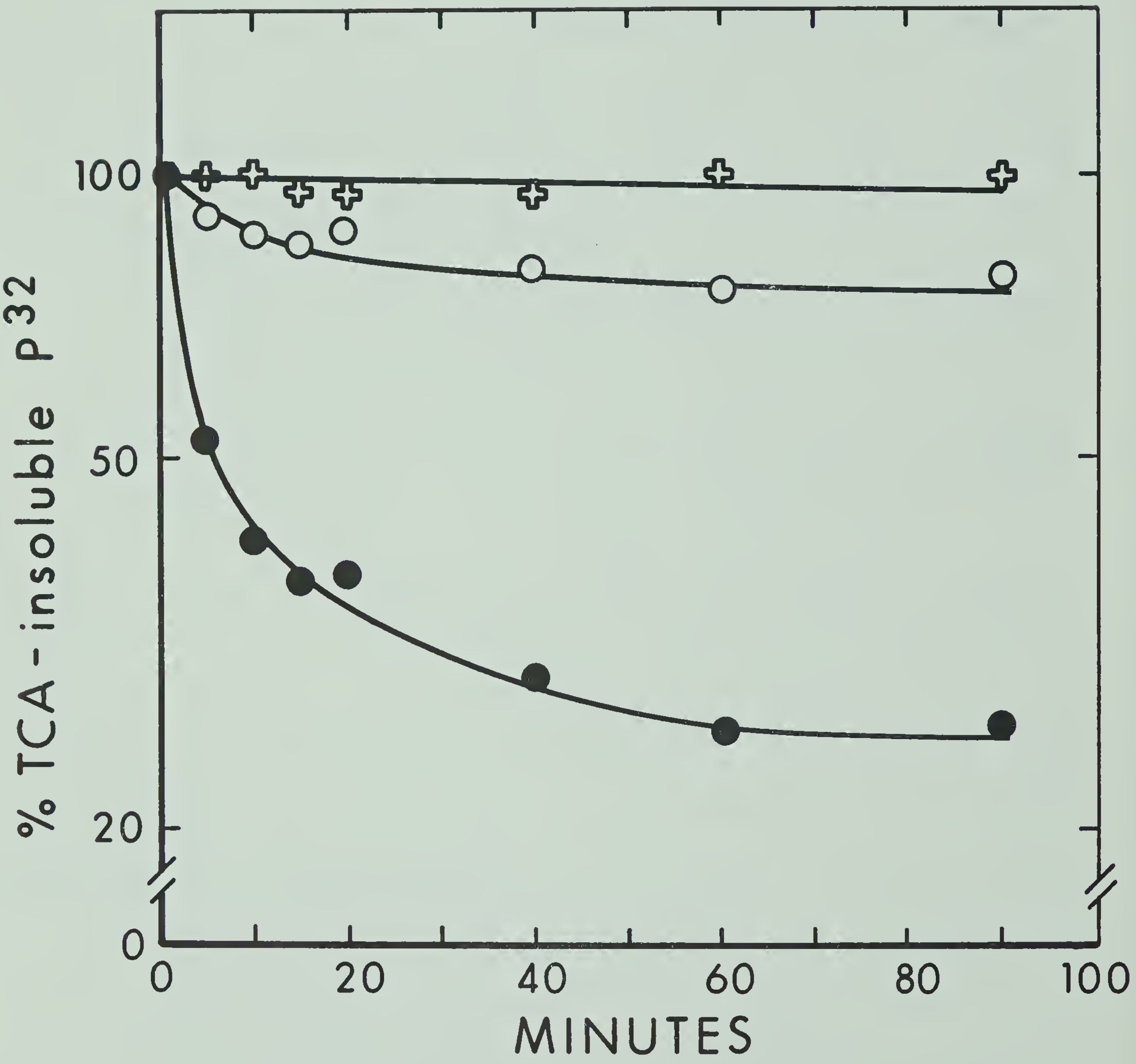

% TCA - insoluble $P^{32}$
100
50
20
0
0
20
40
60
80
100
MINUTES

Figure 5.2. Uncoating of bacteriophage R17 as measured by solubilization of its nucleic acid in TCA by Ribonuclease A

A culture of E. coli K12 was grown to a density of 1 x $10^9$ cells in glucose TMM. CM was added at a final concentration of 100 µg./ml. and cells were chilled in an ice bath. Various portions of the culture were blended and centrifuged, as described in the text, to provide the three "cultures" used: intact cells, a blended culture not subjected to centrifugation, and free F-pili. Ten ml. aliquots of each culture were made to contain 100 µg./ml. ribonuclease, and $P^{32}$-labelled phage R17 was added to each at a multiplicity of one PFU per bacterium. Cultures were assayed at various intervals over a period of 90 minutes for TCA-insoluble radioactivity as described in the text. Control culture (●); blended culture (o); free F-pili (+).

at the time indicated. In the case of the control culture, interaction of the phage with intact bacteria resulted in the hydrolysis of 80% of the labelled phage RNA into acid-soluble material. It is reasonable that this value is not 100%, since in any culture of Hfr bacteria there are at least 20% of the cells at any given time which do not have F-pili (Brinton, 1965). In the case of the blended cells, where no decrease was expected, a decrease of only 20% in TCA-insoluble radioactivity was found. This again was what one might reasonably have expected if there was incomplete removal of F-pili during the blending procedure. When free F-pili were incubated with $P^{32}$-labelled phage, on the other hand, there was no detectable loss of TCA-insoluble material. These results are consistent with the idea that hydrolysis of phage RNA accompanies the uncoating of the phage in this system. It is probable that loss of phage infectivity occurring when R17 and bacteria are incubated in the presence of RNase is a direct result of this hydrolysis.

Considering the short half-life of $P^{32}$ (14.3 days), and the expense of the longer lived isotopes that could be used to label phage, uncoating was normally quantitated in terms of loss of infectivity rather than a decrease in TCA-insoluble radioactivity. The normal assay consisted of placing a 2 ml. sample of the appropriate culture in a

125 ml. Ehrlenmeyer flask, and shaking it on a gyrotory water bath at 37° and 90 rpm. This speed was sufficient to ensure proper mixing, while it avoided excess shearing forces which might cause excessive breakage of F-pili from cells. Purified R17 phage was added to the culture, usually at a multiplicity of 0.01 PFU per bacterium. Low multiplicity permitted the assumption that only one phage was attached per bacterium. If, for example, there were two infectious particles attached per bacterium, the apparent infectivity would be 50% of the input, since two phage on the same cell are in close enough proximity to give rise to a single plaque. At intervals after phage addition, a sample was removed from the culture and diluted into chilled diluent. The diluted samples were mixed with seed bacteria and plated as previously described in Chapter II. Although plaques were visible within six hours, incubation was continued overnight to give the most reproducible results. Where inhibitors were used, these were added to the culture at 10 minutes prior to the addition of the bacteriophage, since this was sufficient time for them to exert their effect on the cell metabolism.

### Preparation of the firefly enzyme

Firefly enzyme used in the luciferin-luciferase photometric determination of ATP was prepared according to

the method of Beutler and Baluda (1964). Firefly lanterns (Sigma FFT) were weighed into a pre-chilled potter homogenizer. Equal amounts of 0.1 M $Na_2HAsO_4$ (pH 7.4) and 0.4 M $MgSO_4$ were added to the dry lanterns to give a final concentration of 20 mg. per ml. The suspension was homogenized for five minutes in an ice bath, using a teflon pestle which was driven by a mechanical stirrer. The homogenate was centrifuged at 10,000 rpm for five minutes in a refrigerated centrifuge, and the turbid yellow-green supernatant containing the luciferase and the luciferin was used as the enzyme. The enzyme stock was chilled in ice overnight before use, since freshly prepared enzyme has been found to give non-specific phosphorescence which decays at an unpredictable rate over the course of the experiment, but which reaches a steady background value after overnight storage. Subsequent storage of the enzyme was at $-20^{o}$, and a preparation was normally discarded after three days. The fact that the frozen and thawed enzyme appeared granular did not affect the results of the assay as long as the solution was mixed well before use.

## ATP assay

(a) Extraction. The determination of the ATP content of bacterial cells in a culture was carried out according to a slightly modified procedure of Cole *et al*. (1967).

Two ml. of bacterial culture were pipetted into a test tube containing 0.5 ml. of chilled $HClO_4$ (30% w/v), mixed briefly on a vortex mixer, and the tube was replaced in ice. After 10 minutes from the time of mixing bacteria with the acid, 1.5 ml. of 1 M KOH was added to the tube. This resulted in the formation of a precipitate of $KClO_4$, which settled by gravity within five minutes. The resulting supernatant solution (pH 1.5 - 2.0) was used in the ATP assay without further treatment.

(b) Assay. All assays of ATP were carried out in the Beckman CPM-100 liquid scintillation counter using the $H^3$-$C^{14}$ module. The coincidence circuit was disconnected, and the rear phototube was used to detect light emission. New scintillation vials were used for the determinations, since it was found that traces of residual scintillation fluid or soap, which had not been removed during the washing procedure, caused a severe quenching of light emitted during the reaction. One - 15 $\lambda$ of the perchlorate extract supernatant was added to a scintillation vial containing 0.9 ml. of 0.044 M Tris buffer (pH 7.4) by means of Drummond Microcap lambda pipettes. The contents of the vial, at a final pH of 7.3, were mixed, and 10 - 20 lambdas of enzyme were added (depending upon its activity). The vial was swirled gently and placed on the elevator of the scintillation

counter. It was positioned in front of the phototube using the manual mode of operation. At 43.5 seconds after the addition of enzyme, the "count" button was depressed and the amount of light emitted over the first 12 seconds was recorded as counts per minute. Standard calibration curves (Figure 5.3), using freshly prepared and spectrophotometrically determined ATP solutions, were prepared for each experiment. The ATP concentration in the standard solution was based on the $A_{257}$ reading using $E_{max.} = 14,700$. The cpm of a blank containing enzyme only were subtracted from each value. Cpm, measured as indicated above, and ATP concentration, were found to be linearly related when the amount of ATP per reaction was in the range of 1 - 15 μμmoles. Reaction mixtures used to construct calibration curves normally did not contain any $KClO_4$, since it was found that this salt, in the amounts present in 15 μl. of extract, had no effect on the reaction. However, when significantly larger amounts of the perchlorate solution were added ($>$100 μl.), there was a noticeable decrease in counts. It was thus important that bacterial cultures were of sufficiently high density that a 15 λ aliquot of the extraction mixture provided sufficient counts. It was found that a culture with an $A_{650}$ reading of 0.25 was adequate to meet these requirements.

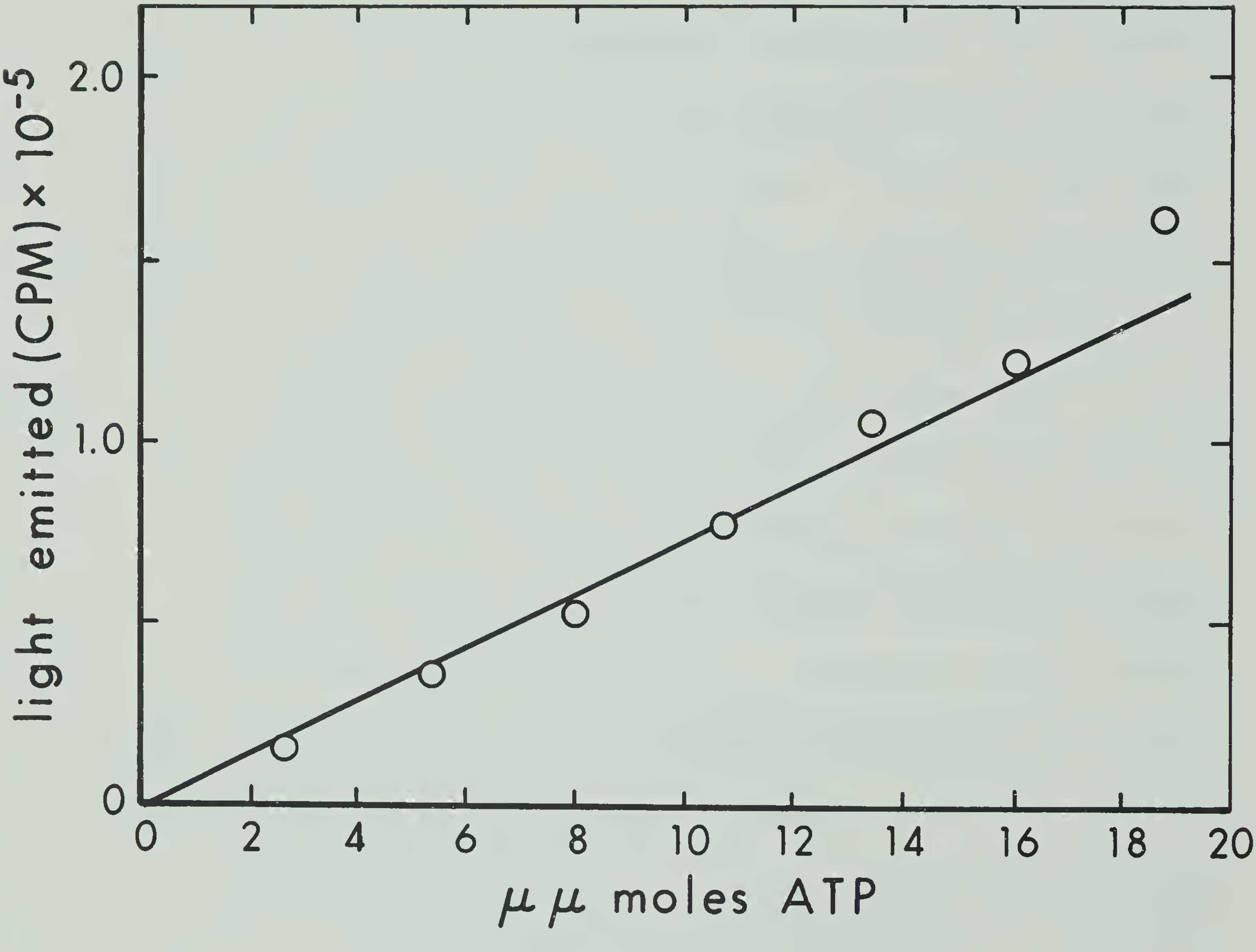

light emitted (CPM) × 10⁻⁵
2.0
1.0
0
0
2
4
6
8
10
12
14
16
18
20
μμ moles ATP

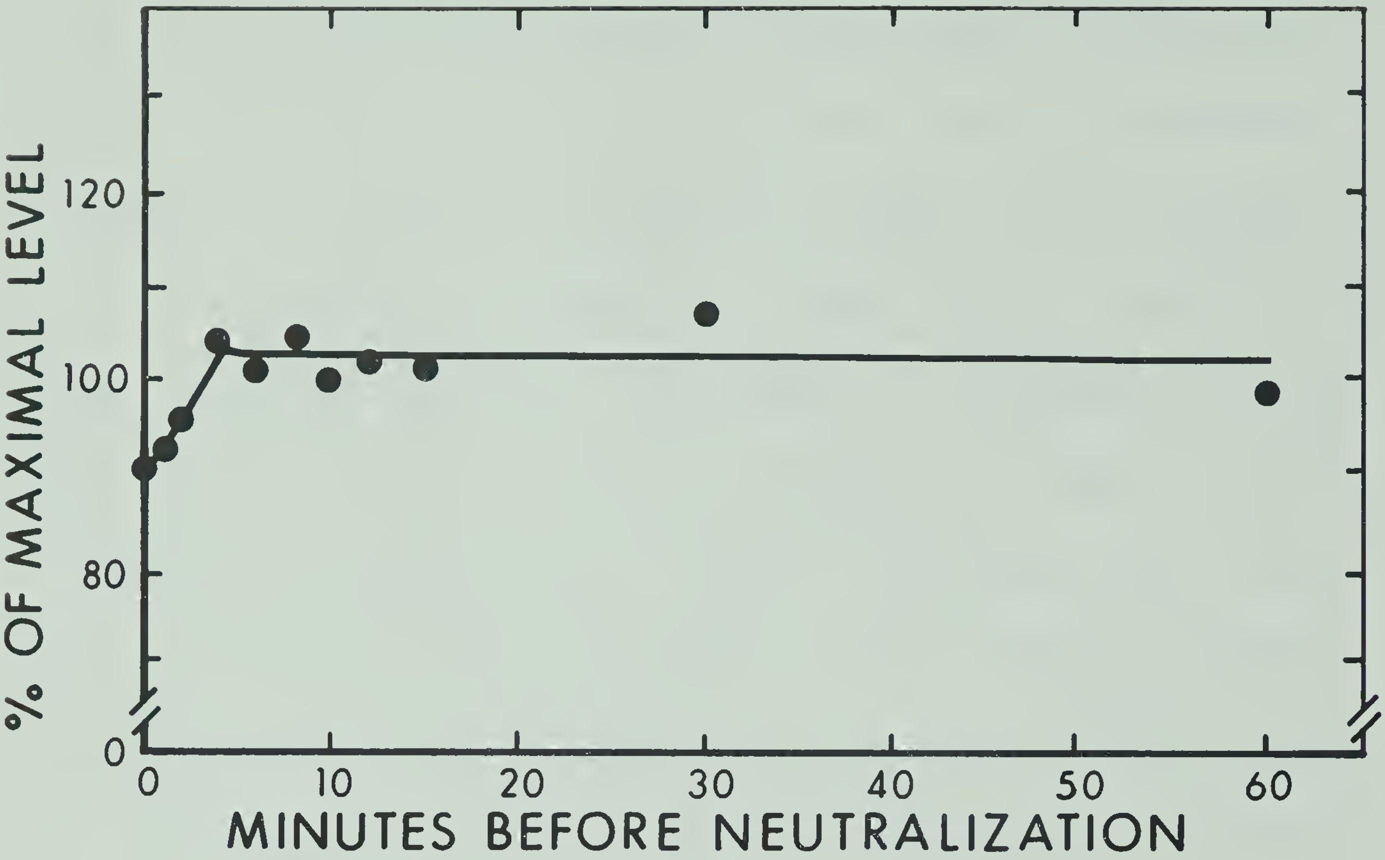
Figure 5.3. Calibration curve for the assay of μμmole quantities of ATP

The indicated amounts of ATP were added to the standard luciferin-luciferase assay mixture as described in the text. Cpm are based on measurements of emitted light during the interval between 45 and 57 seconds after the addition of the enzyme, as measured in the Beckman CPM-100 Scintillation Spectrometer using the rear phototube and the $H^3$-$C^{14}$ module.

The normal ATP assay calls for neutralization of perchloric acid-extracted bacteria after 10 minutes, and the authors suggest that the ATP determinations be made immediately after the $KClO_4$ precipitate settles. It was found, however, that if samples were taken at frequent intervals, it was often not possible to adhere to such a strict schedule. An experiment was therefore designed in which a series of samples of the same culture were acidified simultaneously, but were neutralized at different times. Another set of samples from the same culture were acidified and neutralized at the same time, but ATP determinations were made at different times. The results shown in Figure 5.4 indicated that perchlorate extraction of ATP is essentially complete by 5 minutes. Furthermore, once the extract is neutralized, the ATP is apparently not degraded for at least one hour. These data are only valid for samples which have been stored in an ice bath. It has been reported by the investigators who developed this method that samples can be stored overnight at $-20^{o}$ after neutralization, but it was found that such samples did not always give reliable results.

## Determination of the dry weight of E. coli K12

A culture of *E. coli* K12 was grown in glucose TMM to an $A_{650}$ reading between 0.3 and 0.9. Five or 10 ml. of


% OF MAXIMAL LEVEL
120
100
80
0
0
10
20
30
40
50
60
MINUTES BEFORE NEUTRALIZATION

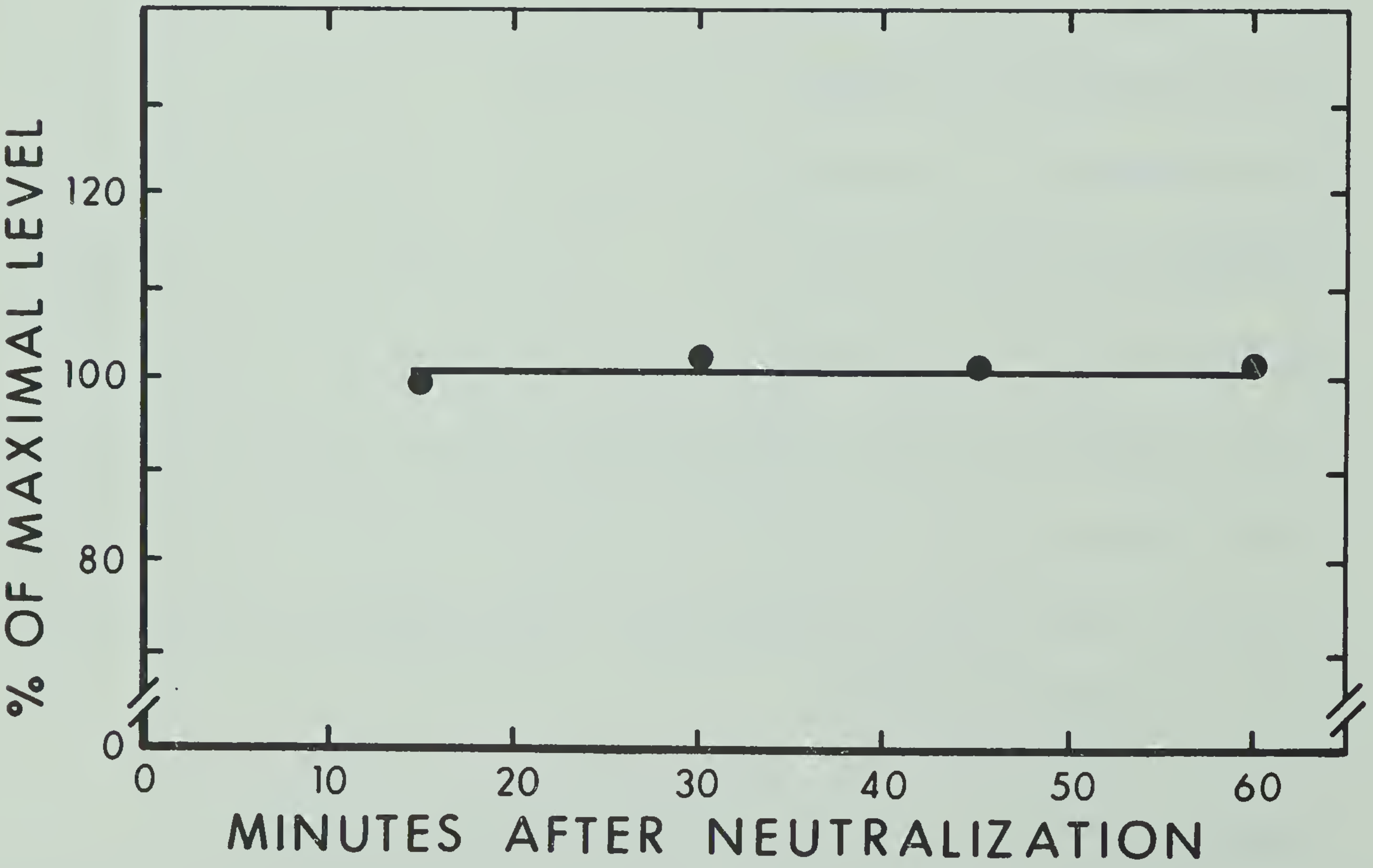

% OF MAXIMAL LEVEL
120
100
80
0
0
10
20
30
40
50
60
MINUTES AFTER NEUTRALIZATION

Figure 5.4 a. The effect of time before neutralization on the extraction of ATP from *E. coli* K12

A culture of *E. coli* K12 was grown to an optical density of 0.4 - 0.6 at 650 mμ. A number of 2 ml. samples were rapidly withdrawn from the culture and added to prechilled tubes containing 0.5 ml. 30% (w/v) perchloric acid. At the indicated times, 1.5 ml. 1 N KOH was added, and the supernatant was assayed after 15 minutes.

Figure 5.4 b. The stability of extracted ATP in the chilled perchlorate supernatant

One of the samples prepared in the experiment described in Figure 5.4 a. was neutralized at 10 minutes. The supernatant was assayed for ATP at intervals up to one hour as described in the text.

culture, or culture medium (in the case of controls), were filtered through Gelman GA-6 cellulose acetate (triacetate) filters which had been pre-weighed with a precision of ± 0.05 mg. Each filter was washed with one 10 ml. volume of 0.85% saline, and the filters were then dried to constant weight by heating in an oven at 250°F. In each experiment, equal numbers of control and experimental filters were used. The average weight of the control filters was subtracted from the weight of the experimental filters to give the average dry-weight of cells contained in the original 5 or 10 ml. of culture. This weight, expressed as a function of the $A_{650}$ value, was found to be 0.37 mg./$A_{650}$ unit, or expressed as a function of cell numbers, 0.27 mg./$10^9$ bacteria. This latter figure is in close agreement with the value for the dry weight of *E. coli* (0.25 mg./$10^9$ cells) reported by Roberts *et al*. (1963).

## The effect of Chloramphenicol and Streptomycin on the ATP levels of E. coli K12

During the course of investigations on uncoating, it was necessary occasionally to employ inhibitors of protein synthesis to block cell division and to prevent the regeneration of pili (Brinton, 1965; Paranchych, unpublished data). If protein synthesis in a cell is blocked, one would expect the utilization, but not the production of ATP

in the cell to be depressed. An inhibitor of protein synthesis could then produce an increase in the intracellular concentration of ATP. This type of agent is best represented by Chloramphenicol (CM). A second inhibitor used in these studies was Streptomycin sulfate (Sm) which, besides acting as an inhibitor of protein synthesis, has other documented side effects. For example, it increases the permeability of bacterial membranes, and, after a long period of time, inhibits respiration of the bacteria (Dubin *et al*., 1963). The ATP level of cells that had been incubated for a prolonged period of time with this drug would, then, be expected to be about the same (or somewhat lower) than the ATP level in control cells.

An experiment was carried out to determine whether these effects were measurable by our techniques. A culture of *E. coli* K12 was grown to an $A_{650}$ value of 0.4 - 0.5 in glycerol TMM. Aliquots were removed for ATP assay and the culture was divided into two 35 ml. aliquots and one 15 ml. aliquot. One 35 ml. aliquot was made 200 μg./ml. with respect to Sm, while to the second 35 ml. aliquot was added the same concentration of CM. The third portion (15 ml.) served as the control. Samples were removed and assayed for ATP levels at various intervals up to 60 minutes, and the results are shown in Figure 5.5. It can be seen that in

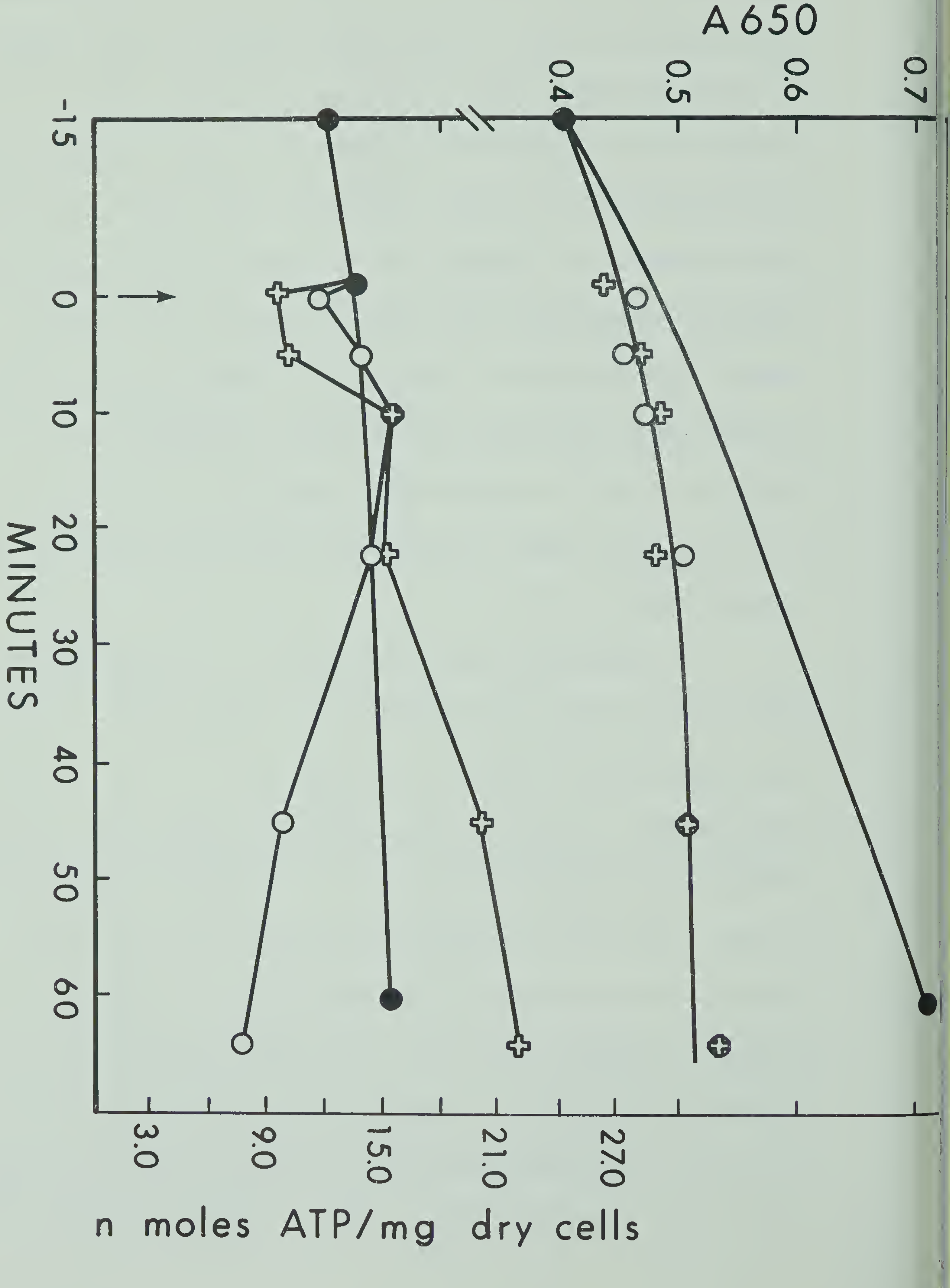

A 650
0.4
0.5
0.6
0.7
-15
0
10
20
30
40
50
60
MINUTES
3.0
9.0
15.0
21.0
27.0
n moles ATP/mg dry cells

Figure 5.5. Time course of the effect of Streptomycin and Chloramphenicol on ATP levels in *E. coli* K12 grown in glycerol TMM

A culture of *E. coli* K12 was grown in glycerol TMM to an $A_{650}$ value of 0.5. At time zero, the culture was divided into three portions: a control culture, a culture containing 200 μg./ml. Chloramphenicol (CM), and a culture containing 200 μg. Streptomycin sulfate (Sm) per ml. Samples were removed at various intervals for assay of ATP and determination of $A_{650}$ values. Control culture (●); 200 μg./ml. Sm (o); 200 μg./ml. CM (+).

both treated cultures there was an initial decline in ATP levels, followed by a fairly rapid recovery. The major effect in both cases can be seen to start at 25 minutes after the addition of the inhibitor. ATP levels rise steadily in the CM-treated culture, while there is a parallel decrease in the culture treated with Sm. The apparent mirror-image relationship between the two experimental curves was not expected and probably has no special significance. It can be seen that the ATP level in the control culture remained relatively constant throughout the course of the experiment. Although the absolute cellular level of ATP is changed in the presence of inhibitors of protein synthesis, the ATP level of such cells should still be able to respond to changes in respiration or glycolysis. Nevertheless, to avoid any complications in evaluating the results of uncoating experiments, neither of these inhibitors was used except where absolutely necessary to prevent the growth of cells.

## Operation of the anaerobic culture flask

Figure 5.6 shows a schematic diagram of the growth flask which was used for studying the uncoating of R17 RNA under anaerobic conditions. The flask assembly comprised a 500 ml. Ehrlenmeyer flask with a side arm containing a self-sealing rubber sleeve stopper, and an all-glass sparger. Bacteria were first allowed to grow to the desired density

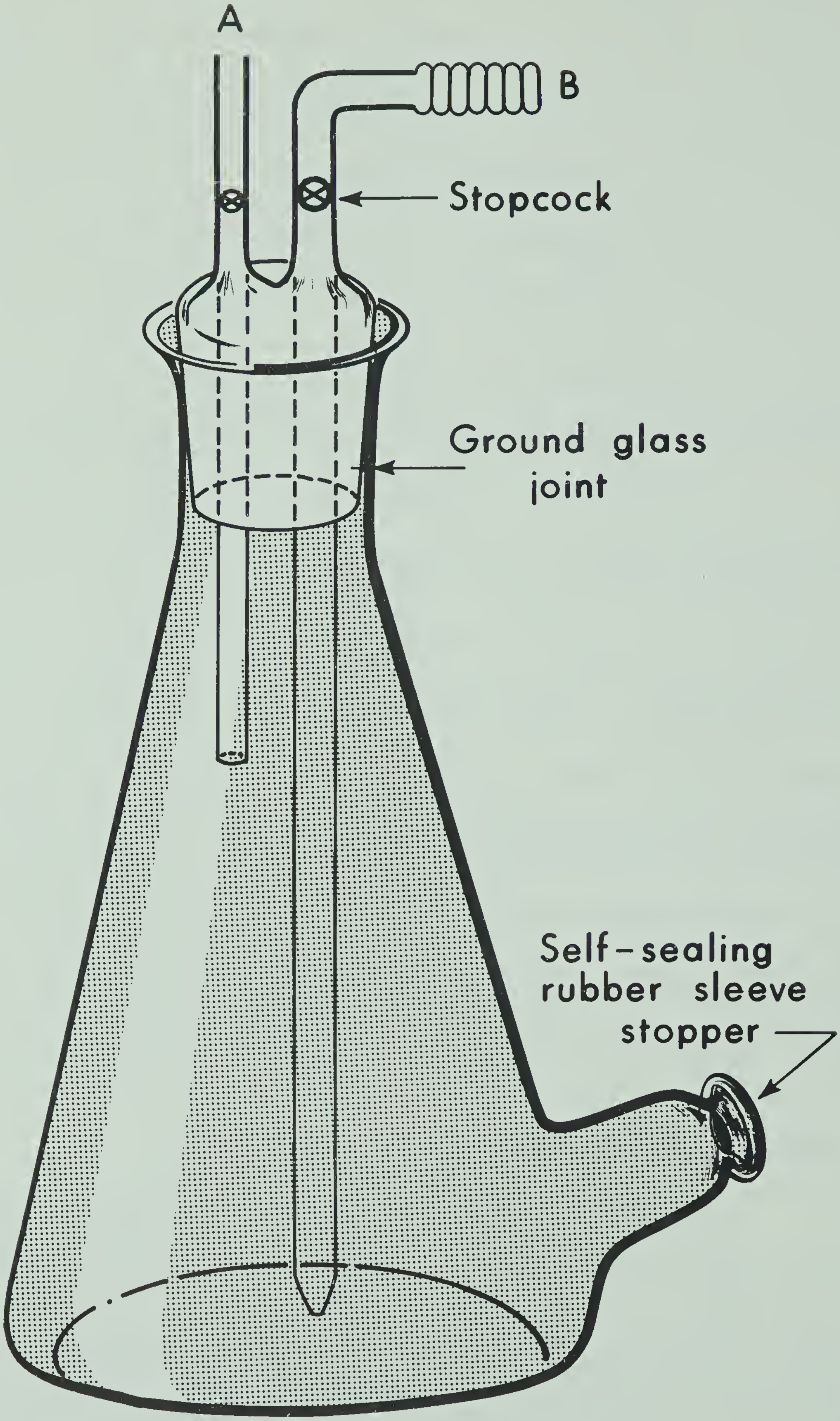

A
B
Stopcock
Ground glass joint
Self-sealing rubber sleeve stopper

Figure 5.6. Schematic diagram of the anaerobic culture flask

Details of the operation of this flask are described in the text.

under normal aerobic conditions, after which the flask was flushed with nitrogen as follows: Portal B was connected by rubber tubing to a tank of 99.997% pure nitrogen (Canadian Liquid Air). Stopcocks A and B were opened, and nitrogen was passed into the flask for one-half minute about one-half inch above the surface of the culture. The stopcocks were then closed and outlet A was attached to a vacuum pump which attained a vacuum of 5 inches of mercury. Stopcock A was then opened and the flask was evacuated for one-half minute. This process of gassing and evacuation was repeated five times, after which nitrogen was allowed to pass freely through the flask at a pressure sufficient to exclude the admittance of air during the course of the experiment. Phage addition and sample withdrawal was accomplished by using a nitrogen-flushed syringe which was inserted through the self-sealing rubber sleeve stopper in the mouth of the side arm. The major advantage of this gassing flask was that the entire culture was exposed to the same atmosphere and was mixed freely at all times, unlike the situation in siphon flasks where a portion of the liquid is always in contact with the external atmosphere.

## Results

### Site of phage uncoating: the effect of blending on the ability of E. coli K12 to cause uncoating of phage R17 RNA

Brinton (1965), and Valentine and Wedel (1965) have shown that the mechanical removal of F-pili from male cells of *E. coli* results in a loss of susceptibility to infection with an RNA phage. Similar results have been obtained in our laboratory with phage R17 using the previously described uncoating assay of radioactive phage. Here, the ability of intact and sheared bacteria to uncoat the phage RNA, as determined by loss of infectivity, was examined. The results obtained are shown in Figure 5.7. It may be seen that blending of a culture of *E. coli* K12, which affected neither viability nor growth rate, resulted in a significant decrease in its ability to reduce the number of infectious units present in the culture, which, as has been previously shown, is probably due to an inhibition of uncoating of the phage nucleic acid. The blending treatment represented by this experiment was accomplished in a Servall Omni-mixer at #50 powerstat setting. Other experiments in which the cells were treated in a Virtis blendor or passed through a #26 gauge hypodermic needle gave essentially the same results. In addition, there seemed to be no apparent correlation between the volume of the culture and the efficiency with

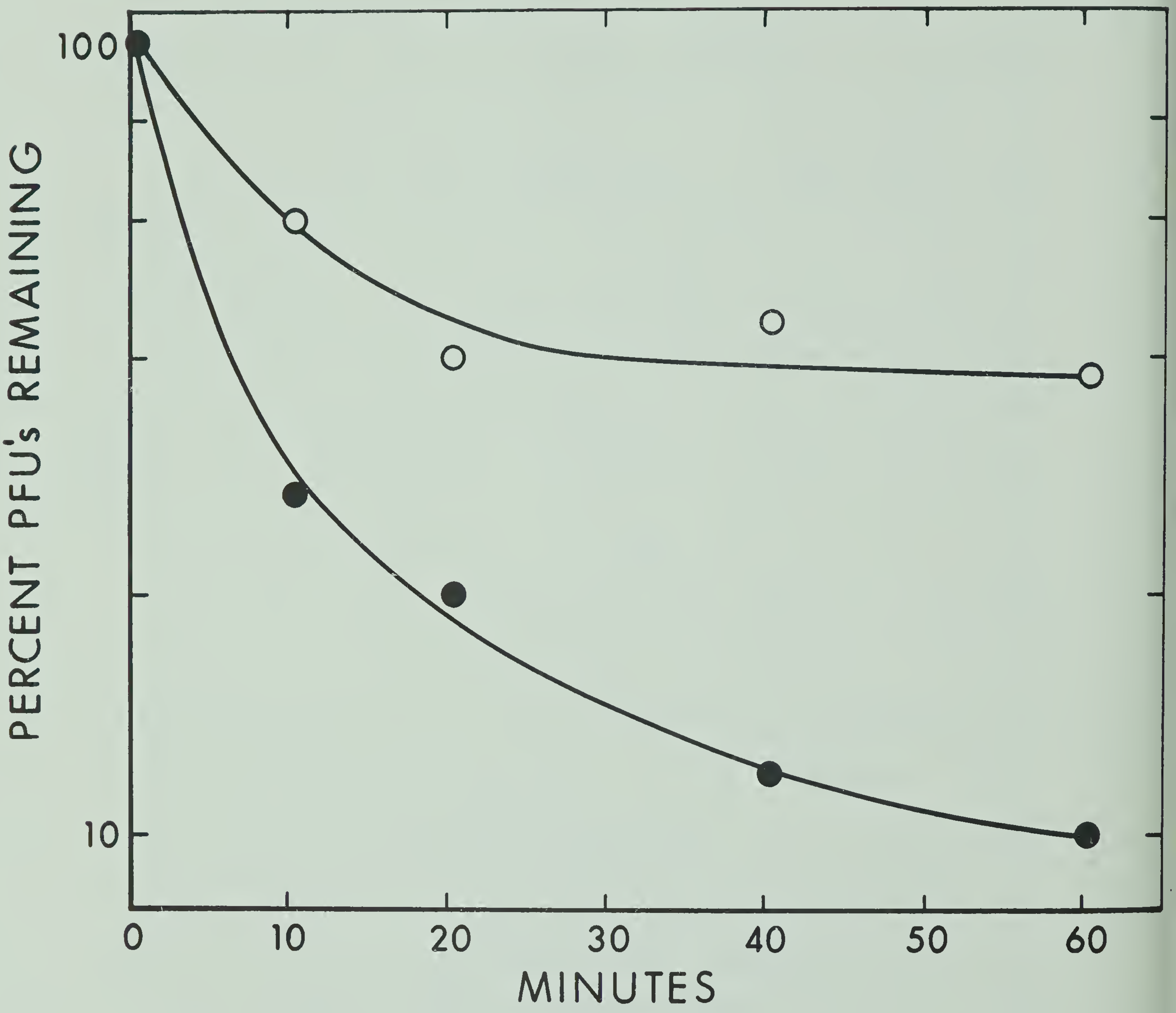

100
10
PERCENT PFU's REMAINING
0
10
20
30
40
50
60
MINUTES

Figure 5.7. Effect of blending on the ability of cells to cause uncoating of phage R17 RNA

A culture of E. coli K12 was grown in glucose TMM to a density of 2 - 3 x $10^8$ bacteria per ml., after which Streptomycin sulfate was added (final concentration = 0.2 mg./ml.) to stop further growth. After 15 minutes incubation with the Streptomycin sulfate, the culture was divided into two portions of 5 ml. each. One portion was blended for one minute in a Servall Omni-mixer at a powerstat setting of 50, while the other received no blending treatment. The two cultures were then assayed for their capacity to uncoat phage RNA as described in Materials and Methods. Unblended culture (●); blended culture (o).

which the F-pili were removed. It was never possible to cause a complete inhibition of uncoating under any of the conditions used, which suggests that none of the blending procedures employed brought about the complete removal of F-pili from the bacterial surface. This observation is in accord with that of Caro and Schnős (1966), who found that male-specific DNA-containing phage fl was able to attach to violently sheared bacteria.

## The relationship of the F-pilus to the cell membrane

During the course of investigations on blending, the idea arose that removal of the cell wall might uncover the base of the F-pilus, exposing more surface on which the phage could be uncoated. From this minor supposition emerged a very interesting idea. If the F-pilus were merely an extension of the normal protoplast membrane, then exposure of this membrane to phage should result in a large increase in the uncoating activity of the cells. To carry this idea one step further, removal of the cell wall of female bacterial cells could be expected to expose a membrane upon which phage could be uncoated. The obvious experiments were performed in which cells were sheared to remove F-pili, then converted to spheroplasts by treatment with Tris-EDTA and lysozyme in hypertonic sucrose. No increase in uncoating was noted in these spheroplasts over that seen with blended,

but non-spheroplasted cells. Furthermore, there was no indication that $F^-$ spheroplasts could uncoat the bacteriophage. It is felt that these experiments provide evidence that the F-pilus, if a membrane or membrane system, is not just an extension of the normal cell envelope. It is readily apparent that these data provoke a number of interesting questions regarding the nature of the F-pilus and its relationship with the rest of the cell surface. In 1965, Brinton proposed that the F-pilus grows out specifically at the site of the F-DNA segment. The evidence discussed here is certainly consistent with such an idea, although it does not provide any direct experimental support.

### The effect of temperature on the uncoating of R17 RNA

One of the characteristic features of normal reactions, both chemical and biological, is the variation in reaction rate with changes in temperature. There are a number of studies in which investigators have looked at the effect of low temperature on the infection of bacterial cells by RNA bacteriophage. According to Brinton (1965), and Valentine and Wedel (1965), the transport of the RNA within the F-pilus, and its subsequent "penetration" into the body of the bacterial cell is suppressed at $0^{\circ}$. This fact is further substantiated by the data of Knolle (1967) which showed that low temperatures reduce "invasion" of the

cell by the phage genome more than they affect the attachment stage. If the uncoating process is an energy-requiring reaction, it should vary in a highly predictable manner with temperature, and the calculated energy of activation for the uncoating reaction should be in the range of that provided by the hydrolysis of one of the high energy phosphates. To test this hypothesis we followed the normal protocol for an uncoating experiment. A single culture of K12 was divided into a number of 2 ml. aliquots, which were equilibrated for 10 minutes at the experimental temperature before the addition of the bacteriophage. The results, presented in Figure 5.8, show that a graded response to temperature was indeed obtained. It is important to note that cold-inhibition of uncoating is completely reversible, for it may be seen in Figure 5.9 that a culture which had been at 0° for 60 minutes began to uncoat phage immediately upon being placed at 37°.

An experiment of the type outlined above lends itself to an estimation of the energy of activation for the uncoating process. Using the integrated Arrhenius equation:

$$\ln \frac{k_1}{k_2} = \frac{A}{R} \left( \frac{T_2 - T_1}{T_1 T_2} \right)$$

where the rate constants $k_1$ and $k_2$ represent the per cent loss of phage per minute over the first 10 minutes at 21° and 37°, the energy of activation of uncoating can be

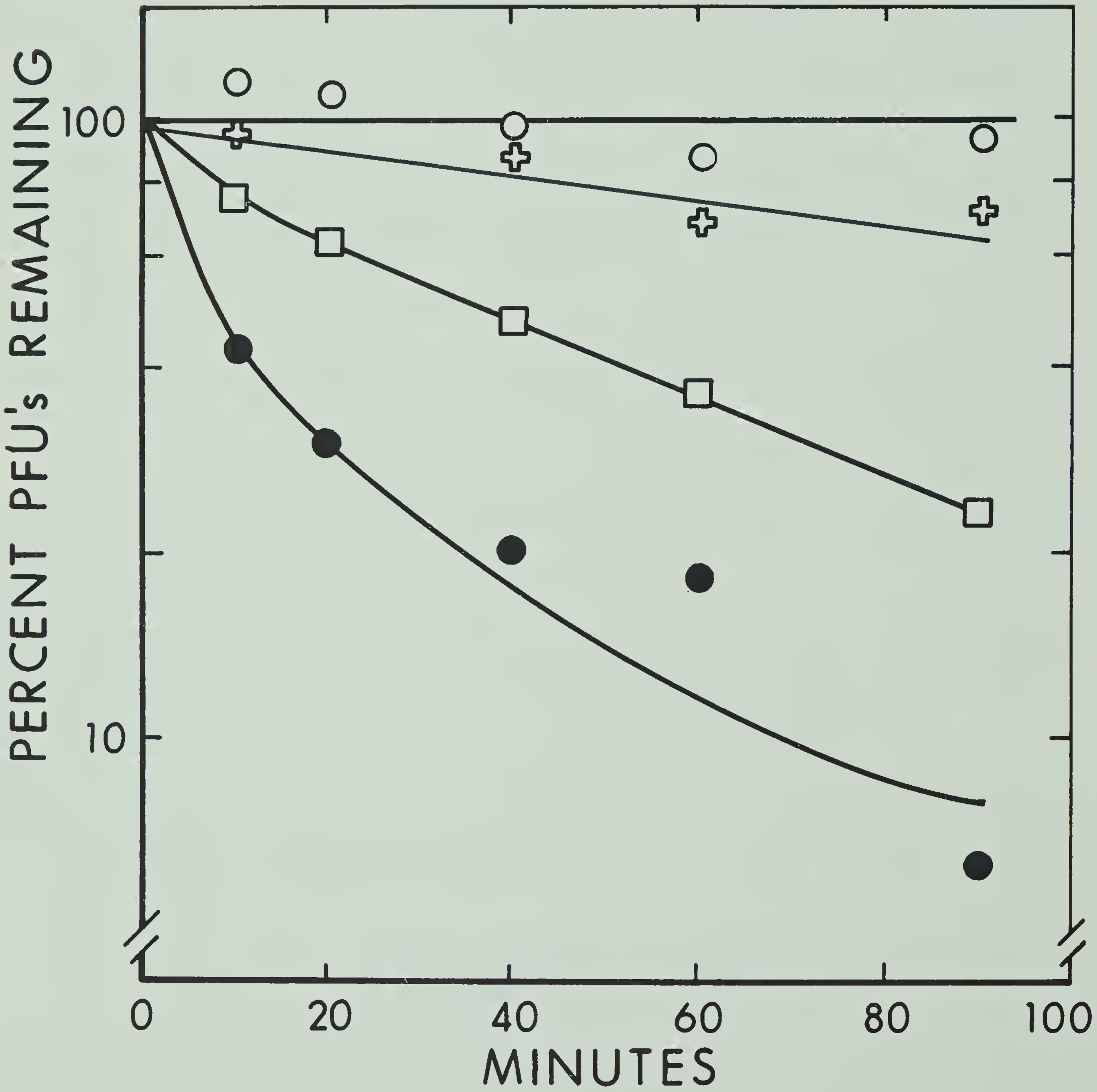

PERCENT PFU's REMAINING
100
10
0
20
40
60
80
100
MINUTES

Figure 5.8. Effect of temperature on uncoating of R17 RNA by *E. coli* K12

A culture of *E. coli* K12 was grown in glucose TMM to a density of 4 x $10^8$ bacteria/ml. after which RNase and Sm were added to give final concentrations of 100 μg./ml. The culture was divided into 10 ml. aliquots which were equilibrated at 10, 20, 30 and $37^o$. R17 phage was added to each culture at a multiplicity of $10^{-3}$ PFU's per bacterium, and each culture was then assayed for its capacity to uncoat phage RNA as described in Materials and Methods. $10^o$ (o); $20^o$ (+); $30^o$ (□); $37^o$ (●).

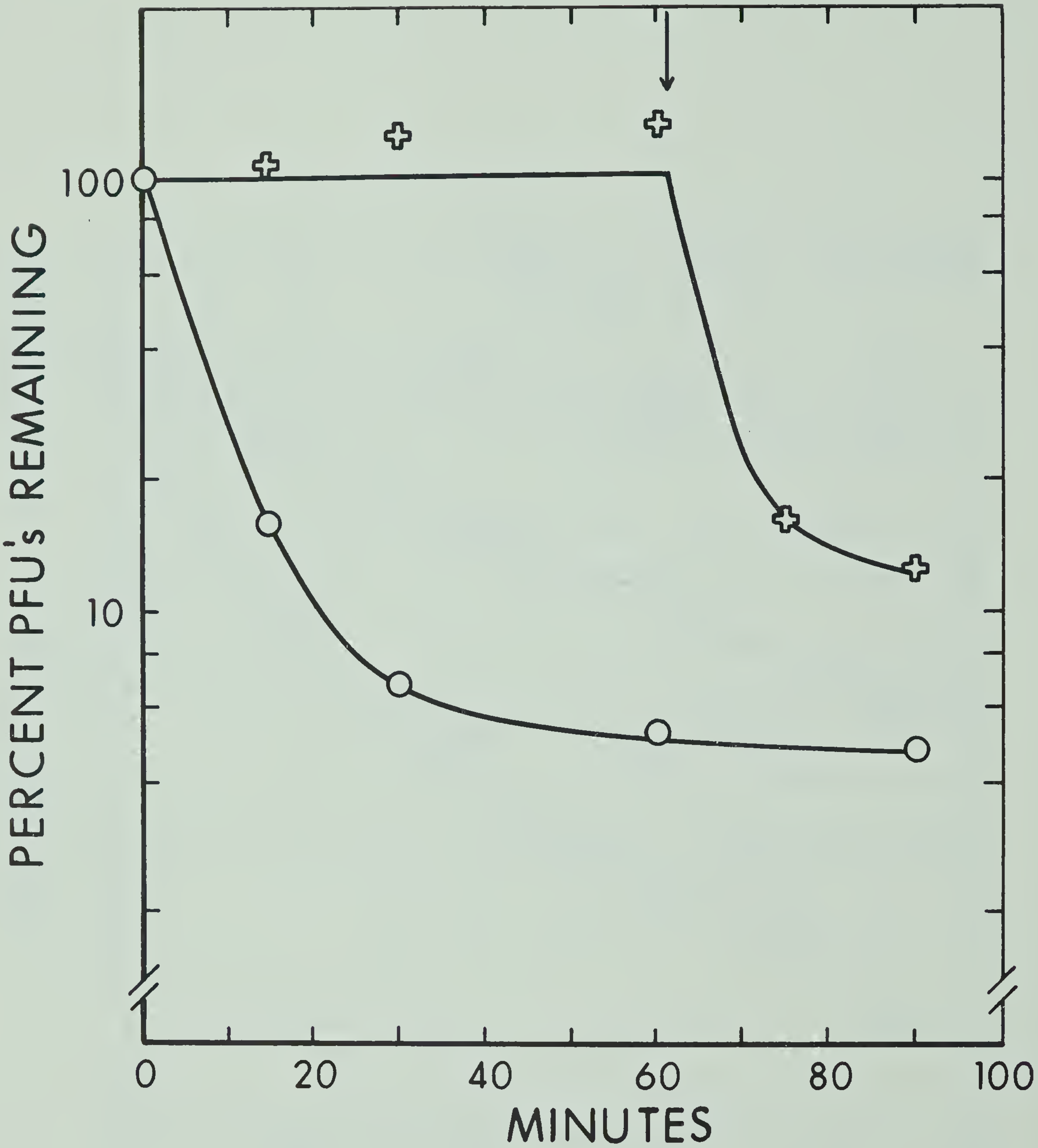

PERCENT PFU's REMAINING
100
10
0
20
40
60
80
100
MINUTES

Figure 5.9. The effect of prechilling on the ability of *E. coli* K12 to uncoat R17 RNA

A culture of *E. coli* K12 was grown in glucose TMM to a density of 1 - 2 x $10^8$ bacteria/ml. The culture was made 100 μg./ml. with respect to ribonuclease and Sm. One portion of the culture was incubated at 37°, a second portion was incubated at 0°. Both aliquots were infected with R17 at a multiplicity of 0.1 PFU/bacterium. Samples were removed from each culture at intervals over a period of 60 minutes for assay of infectivity. At 60 minutes, the chilled culture was transferred to a 37° water bath and additional samples were taken from both cultures for assay of PFU's. Control culture (o); prechilled culture (+). Arrow indicates time of transfer of prechilled culture to a 37° water bath.

calculated to be roughly 10,000 cal. mole$^{-1}$. Many biological reactions have a $Q_{10}$ of 2, for which the activation energy would be 11,420 cal. mole$^{-1}$. These data are therefore consistent, with the premise that uncoating is a typical energy-requiring biological process. Although low temperature inhibition of uncoating is highly suggestive of an energy requirement, it was felt that a more direct approach would be to carry out uncoating in the presence of a number of metabolic inhibitors, and to correlate the uncoating ability of a bacterial cell with its overall energy state as reflected by the level of its acid-soluble ATP pool. Among the substances investigated with respect to their ability to alter the uncoating ability of cells were nitrogen, sodium arsenate, 2,4-dinitrophenol, and 2-deoxy-D-glucose.

The decision to use the measurement of ATP as an index of the overall energy state of the cell was based upon several considerations. Of prime concern was the necessity of measuring a substance which participates in a large number of different reactions, but whose function, in most cases, is energy donation rather than biosynthesis. It can be seen from the literature that ATP fulfills this function with an ubiquity unlike that of any other cellular component. The reader is asked to consult Racker (1965) for an excellent

review of this subject. A second requirement for an energy indicator was that it occurs in measurable amounts, and that the method for its assay be simple and reproducible as well as adaptable to small volumes of a bacterial culture. Disregarding the compounds which are formed via activation or complexing with ATP, a major supply of high-energy compounds is the nucleoside triphosphate fraction of the cold-TCA-soluble nucleotide pool, which has the following composition under balanced growth in a glucose-supplemented minimal medium (Roberts *et al*., 1955):

| | μmoles P/gram dry weight |
|---|---|
| $PO_4$ | 80 |
| ATP | 20 |
| ADP | 12 |
| GTP | 7 |
| GDP | 7 |
| UTP | 15 |
| UDP | 20 |
| CTP | 5 |
| CDP | 5 |

It can be seen that of the nucleoside triphosphates, the most abundant compound is ATP, although UTP is also present in considerable quantities. It would be desirable to assay for ADP, AMP, UTP, CTP and GTP, but this is not possible because no simple sensitive assay methods are available for compounds other than ATP, and, as will be shown below, manipulations involved in concentrating the bacteria cause

changes in the level of high energy compounds. The measurement of ATP is possible, however, since its assay was made rapid and sensitive with the introduction of the luciferin-luciferase reaction (Strehler, 1957). This method is based on the measurement of an ATP-induced light emission in extracts of firefly lanterns. Under the appropriate conditions, the intensity of the emitted light is directly proportional to the concentration of ATP. A scintillation phototube is used as a sensitive receptor for the detection of emitted light, and it is found that the scintillation counter records light from samples containing as little as 1 μμmole of ATP. This makes it possible to use as little as 2 ml. of the bacterial culture, and eliminates the need to concentrate bacteria by centrifugation (see the general methods section of this chapter for complete extraction procedures). Using this method, the average amount of ATP present in *E. coli* cells grown under normal conditions was found to be approximately 14 nmoles/mg. dry weight. If, however, cells were centrifuged and resuspended once prior to extraction, as is necessary with less sensitive methods of assay to concentrate the substance to be measured, it was found that the ATP levels of these cells were consistently about 0.4 nmoles/mg. dry weight. This same drop in ATP content was also observed by Cole *et al*. (1967). This

35-fold decrease, resulting simply from centrifuging and resuspending the bacteria once, makes it impracticable to assay for ATP unless a method is used which avoids centrifugation. It is likely that many, if not all, high energy compounds would show a parallel decrease as a result of similar treatment. The conclusions that can be drawn from these data are that, besides being the energy source for a number of cellular reactions, ATP occurs in relatively high concentrations and can be rapidly extracted and assayed with a significantly greater sensitivity than can be obtained with any other energy-donating compound.

These, therefore, are the considerations that led to the use of ATP in this study as an index of overall energy. It is important, however, that constant reference to ATP should not create the impression that this is the compound which has been proven to specifically provide the energy to cause uncoating of the phage nucleic acid, although it may well be so.

### The effect of anaerobiosis on uncoating of R17 RNA

The bacterium *E. coli* derives the majority of its ATP from the energy-yielding processes of glycolysis and respiration. Forty moles of ATP are normally generated per mole of glucose completely metabolized to $CO_2$ and $H_2O$. This is the net yield disregarding the two moles of ATP

required for glucose phosphorylation. Glycolysis furnishes eight moles of ATP, two from substrate phosphorylation in the conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid, and in the dephosphorylation of enolphosphopyruvic acid to enolpyruvic acid. A further six moles of ATP are derived from the oxidation of reduced NAD through the flavoprotein cytochrome system. This NADH is formed during the oxidation of phosphoglyceraldehyde. The further oxidation of pyruvate to $CO_2$ and water, provides the additional 30 moles of ATP (actually 29 moles of ATP and one mole of GTP). Glycerol can presumably be metabolized via the same pathway after phosphorylation to $\alpha$-glycerophosphate. Anaerobiosis then, by inhibiting respiration, would theoretically prevent the production of three-fourths of the ATP normally generated during the catabolism of glucose (or glycerol). The actual yield of ATP under anaerobic conditions may be slightly higher than this, since glycolysis is stimulated in the absence of oxygen (Crabtree Effect). It should therefore be possible to lower intracellular levels of ATP by subjecting bacterial cells to an oxygen-free atmosphere.

An experiment designed to examine this premise and to determine the effect of anaerobiosis on the ability of bacterial cells to uncoat phage was performed. A 90 ml.

culture of E. coli K12 was grown in glycerol TMM to a cell density of 2 x $10^8$/ml., at which point RNase (final concentration = 100 μg./ml.) was added and half the culture was transferred to the special $N_2$ gassing flask for evacuation and atmosphere exchange. The remaining 45 mls. were left in a standard aerobic culture flask. Samples were taken before and after the atmosphere exchange step for ATP assays. Both cultures were then infected with R17 phage at a multiplicity of 1 PFU/bacterium, and for the ensuing 60 minutes, samples were removed at intervals for assays of infectivity, ATP, and absorbance at 650 mμ. Air was readmitted to the nitrogen flask at 50 minutes post-infection (air pressure was approximately the same as that of the nitrogen), and additional samples were taken from both flasks for the determination of PFU's, ATP, and absorbance at 650 mμ.

The results depicted in Figure 5.10 show the response of the cellular ATP levels to this treatment. It may be seen that anaerobiosis caused a decrease in the level of ATP from 12.5 to 8.7 nmoles/mg. dry weight of cells during the first 10 minutes of $N_2$ gassing, indicated by the shaded region of the figure. Thereafter, the ATP level continued to decrease to approximately 7.5 nmoles/mg., where it remained for approximately 40 minutes with a slow recovery to 8.6 nmoles/mg. by 50 minutes. The readmission of air caused a rapid

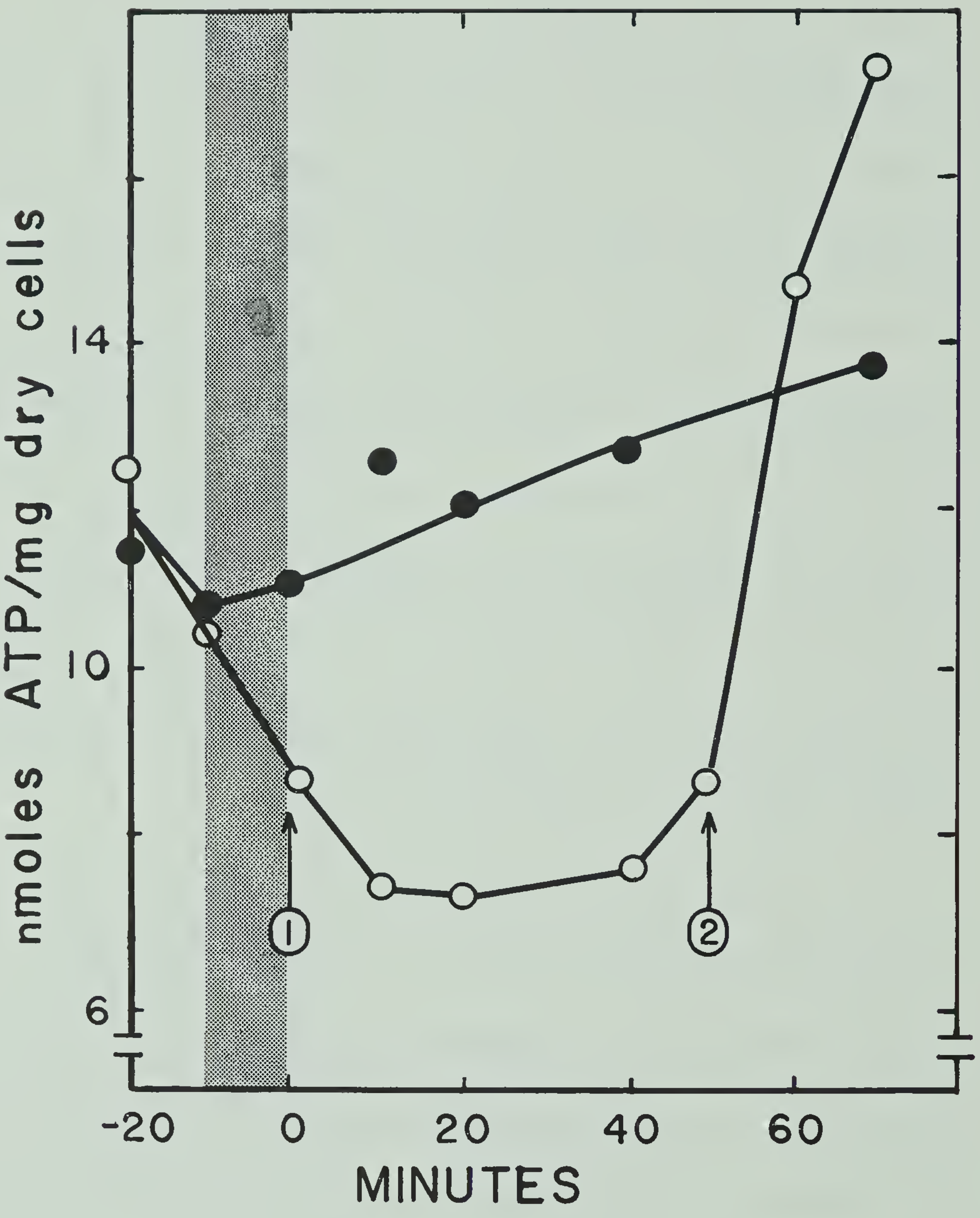

nmoles ATP/mg dry cells
14
10
6
-20
0
20
40
60
MINUTES
①
②

Figure 5.10. The effect of anaerobiosis on ATP levels in *E. coli* K12

The same culture and conditions were used for this experiment as are described in the legend to Figure 5.11, except that samples were removed prior to nitrogen gassing to establish control levels in both flasks. At each time interval, a 3 ml. sample was removed, 1 ml. of which was used to measure absorbance at 650 mμ, and the other 2 ml. were rapidly added to cold $HClO_4$ for extraction of ATP. Samples from the KOH neutralized perchlorate extract were assayed for ATP by the method outlined in detail in the Materials and Methods section of this chapter. The shaded area represents the period of initial atmosphere exchange in the anaerobic culture flask (after this time, nitrogen flow was constant until the readmission of air, indicated by arrow ②). Arrow ① indicates the time at which phage R17 was added to the culture. Aerobic culture (●); anaerobic culture (o).

rise to 13.6 nmoles/mg. In contrast with these results, the ATP level in the control culture remained fairly constant at about 12 - 13 nmoles/mg. The accompanying results, pictured in Figure 5.11, show that uncoating was reduced by 75% during the period of anaerobiosis, whereas the readmission of oxygen resulted in a simultaneous increase in the ATP levels and in the degree of uncoating. It is especially interesting to note the absence of a lag period in the uncoating reaction. The fact that phage uncoating begins immediately upon the readmission of air to the system indicates that nitrogen gassing probably did not cause depiliation of the cells. (The gas was passed into the flask above the culture medium.) If depiliation had occurred, it would have taken from 5 to 10 minutes for the regrowth of F-pili to occur in order that uncoating could take place (Brinton, 1965).

When the effect of anaerobiosis on the uncoating of phage RNA was examined in glucose TMM (the previous results were for cultures grown in glycerol TMM), the data concerning ATP levels were found to be essentially identical to those shown in Figure 5.10, but only a very slight inhibition of uncoating was observed. The significance of this observation will be discussed in the final portion of this chapter.

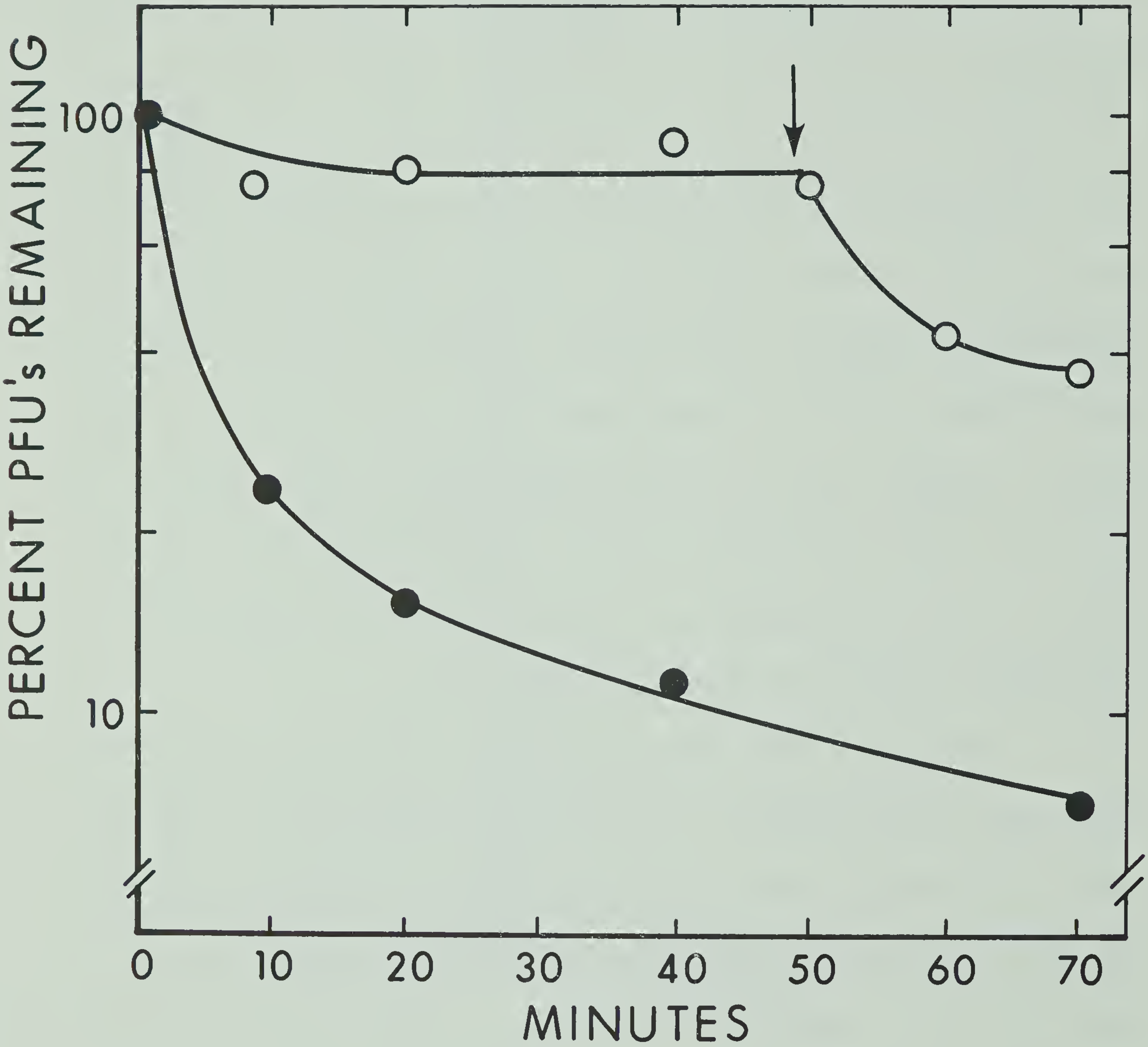

PERCENT PFU's REMAINING
100
10
0
10
20
30
40
50
60
70
MINUTES

Figure 5.11. The effect of anaerobiosis on uncoating of R17 RNA by *E. coli* K12

A culture of *E. coli* K12 was grown in glycerol TMM to a density of 2 x $10^8$ bacteria/ml., at which time RNase was added to a final concentration of 100 $\mu$g./ml. After a further incubation for 15 minutes, one-half the culture was made anaerobic. A constant flow of nitrogen through this flask was maintained for the period 0 - 50 minutes post-infection. Phage R17 was added to both cultures at a multiplicity of one PFU/bacterium at time zero, and samples were removed at intervals for infectivity. In the case of the anaerobic flask, the addition of and the withdrawal of samples was accomplished by means of a syringe inserted in the rubber sleeve stopper of the special side arm. At 50 minutes post-infection (second arrow in Figure 5.10), air was admitted to the previously anaerobic culture and additional samples were removed for infectivity assay. Aerobic culture (●); anaerobic culture (o).

## The effect of 2,4-dinitrophenol on the uncoating reaction

Anaerobiosis inhibits respiration by blocking the normal operation of the electron transport chain. Other substances which allow electron transport to occur normally (and often at increased rates), can prevent the coupling of the redox reactions to the phosphorylation of ADP, thus preventing the formation of ATP. These substances are called uncouplers rather than inhibitors, although the net result of their action upon the cell is still a decrease in the level of cellular ATP. The effect of one of the more commonly studied uncoupling agents, 2,4-dinitrophenol, on the uncoating reaction was studied.

It was found that this compound did inhibit the uncoating reactions, although, for reasons unknown, the degree of inhibition varied markedly from experiment to experiment. The data showed that although DNP caused comparable decreases in the ATP levels of glycerol TMM and glucose TMM-grown cells, its effect on the uncoating reaction was less marked in the latter type of culture.

## The effect of sodium arsenate on the uncoating of R17 RNA

Arsenate, besides uncoupling respiratory chain phosphorylation (Crane, 1953), is capable of uncoupling the substrate-linked phosphorylation associated with phosphoglyceraldehyde and $\alpha$-ketoglutarate oxidation (Needham, 1937;

Sanadi, 1954). Another effect of arsenate is its ability to arsenolyze (hydrolyze) acetyl phosphate. This latter compound is an important high-energy compound of the acyl phosphate type (Huennekens and Whiteley, 1960), which in bacteria, may be important in the synthesis of ATP. In addition, acetyl phosphate can be used directly to phosphorylate compounds normally phosphorylated by ATP (Green and Meyerhof, 1952).

One of the normal routes of acetyl phosphate biosynthesis is via the following reaction (catalyzed by the enzyme phosphotransacetylase):

$$\text{Acetyl-CoA} + P_i \rightleftharpoons \text{Acetyl Phosphate} + \text{CoA}$$

If arsenate is present in this reaction, acetyl arsenate, an unstable compound, is formed and readily becomes hydrolyzed to acetyl-CoA and arsenate (Huennekens and Whiteley, 1960). This reaction gives arsenate a definite apyrase action which might be expected to lower cellular ATP levels.

Arsenate was therefore tested for its ability to alter ATP levels and to inhibit uncoating. Two ml. aliquots of a culture of E. coli K12 in glycerol were incubated in the presence of various concentrations of sodium arsenate for 10 minutes, a period of time sufficient for arsenate to exert its maximum effect. R17 phage and RNase were then added to one of each pair of samples, and the loss of PFU's

was followed in the usual manner. The duplicate sample was used for the assay of ATP. The data are shown in Figure 5.12.

It may be seen that uncoating was completely inhibited by sodium arsenate concentrations of 0.01 M or greater, and that 0.01 M arsenate produced a decrease in the ATP content of the cells from the control level of 14 nmoles/mg. dry weight to 1.3 nmoles/mg. dry weight. Lower concentrations of sodium arsenate produced a lesser effect on both uncoating and intracellular ATP levels.

When an experiment identical to this one was carried out using cells which had been grown in glucose TMM, the results (Figure 5.13) were clearly different. Even at concentrations of sodium arsenate as high as 0.1 M, uncoating was inhibited to the extent of only 14%, although the ATP content of these cells was reduced to less than 20% of the level in the control cells.

In order to provide a more convenient, visual illustration of the effects of Na arsenate on ATP levels in the uncoating of R17 phage by cells grown in glycerol TMM and glucose TMM media, the data summarized in Figures 5.12 and 5.13 has been replotted in the following two figures.

From Figure 5.14 it may be seen that Na arsenate causes a decrease in the ATP levels of cells grown in either

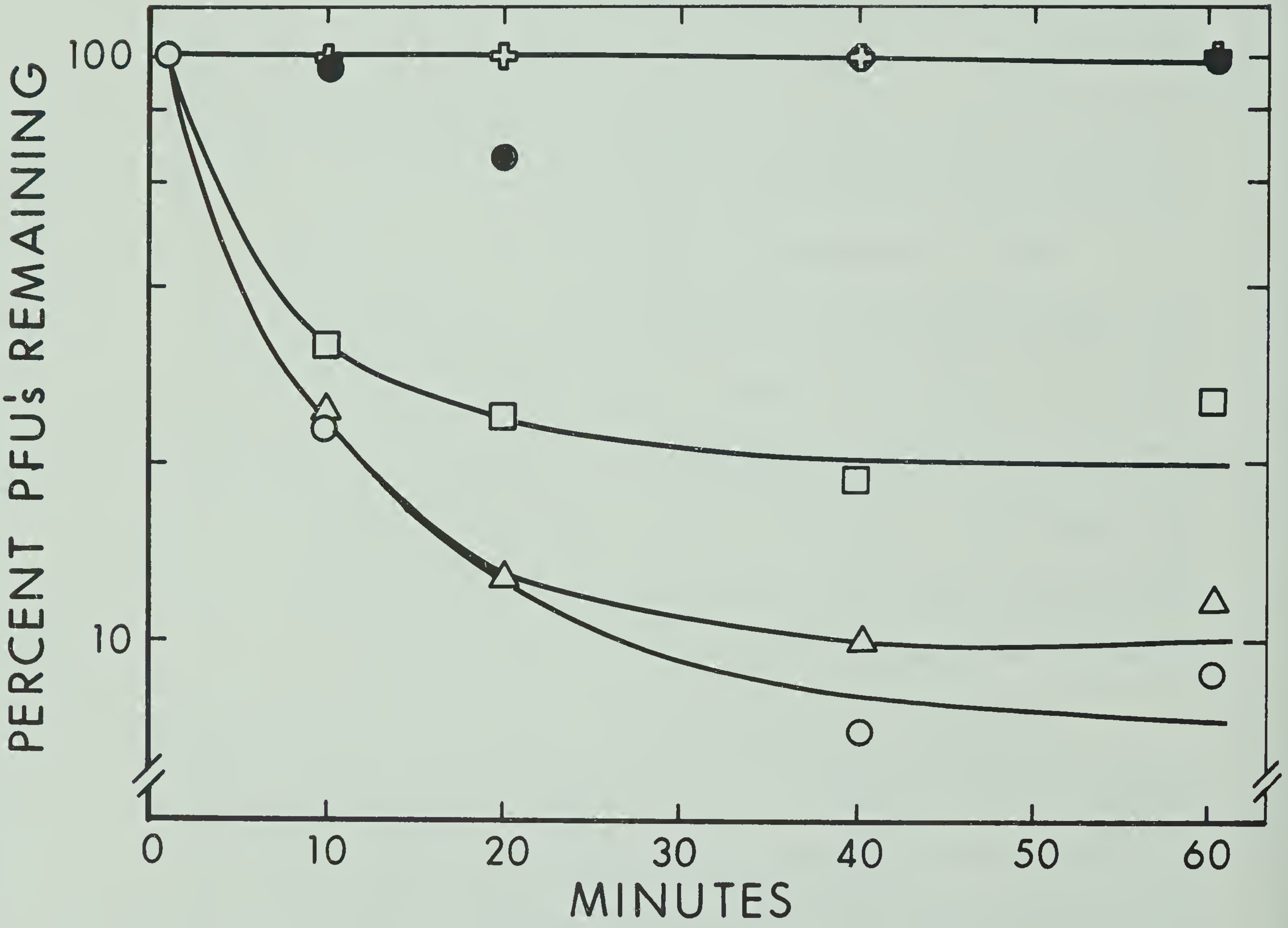

PERCENT PFU's REMAINING
100
10
0
10
20
30
40
50
60
MINUTES

Figure 5.12. The effect of sodium arsenate on the ability of *E. coli* K12 (grown in glycerol TMM) to uncoat R17 RNA

A culture of *E. coli* K12 was grown to a density of $3 \times 10^8$ bacteria/ml. in glycerol TMM. Cultures were made 100 μg./ml. with respect to ribonuclease, and 2 ml. aliquots of the culture were incubated for 10 minutes with the indicated concentrations of sodium arsenate. R17 phage was added to each incubation mixture at a multiplicity of 0.01 PFU per bacterium, and samples were withdrawn at intervals for infectivity assays. Other aliquots of the same culture were incubated for 10 minutes with the same concentrations of sodium arsenate, and were assayed for absorbancy at 650 mμ and ATP content as described in the Methods and Materials section of this chapter. Control culture - 14.4 nmoles ATP/mg. (o); $1 \times 10^{-3}$ M arsenate - 8.3 nmoles ATP/mg. (△); $3 \times 10^{-3}$ M arsenate - 5.1 nmoles ATP/mg. (□); $1 \times 10^{-2}$ M arsenate - 1.3 nmoles ATP/mg. (●); $1 \times 10^{-1}$ M arsenate - 0.3 nmoles ATP/mg. (+).

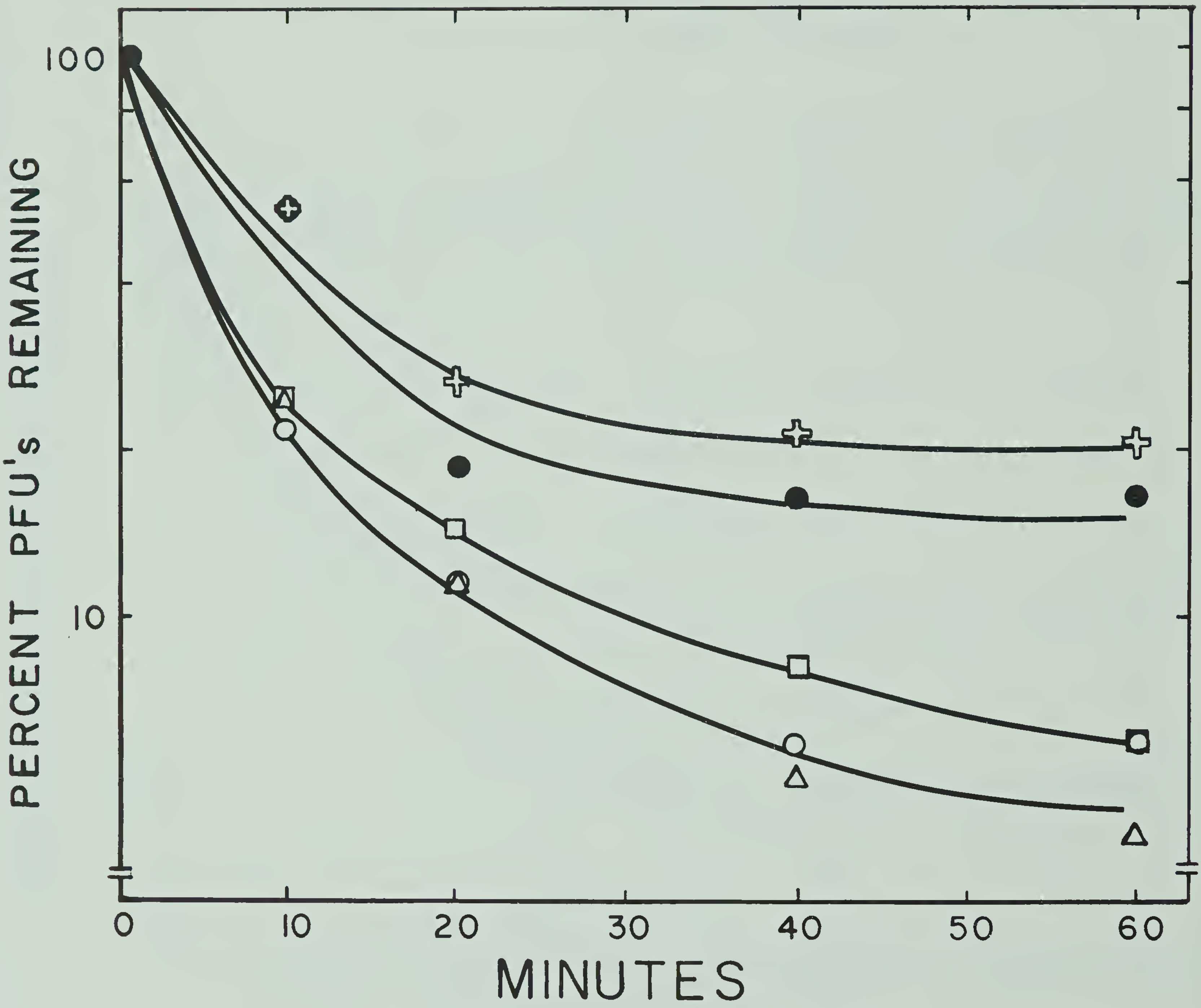

100
10
PERCENT PFU's REMAINING
0
10
20
30
40
50
60
MINUTES

Figure 5.13. The effect of sodium arsenate on the ability of *E. coli* K12 (grown in glucose TMM) to uncoat R17 RNA

Details of this experiment are given in the legend to Figure 5.12. Control culture - 14.4 nmoles ATP/mg. (o); 1 x $10^{-3}$ M arsenate - 12.3 nmoles ATP/mg. (△); 3 x $10^{-3}$ M arsenate - 4.9 nmoles ATP/mg. (□); 1 x $10^{-2}$ M arsenate - 2.9 nmoles ATP/mg. (●); 1 x $10^{-1}$ M arsenate - 2.3 nmoles ATP/mg. (+).

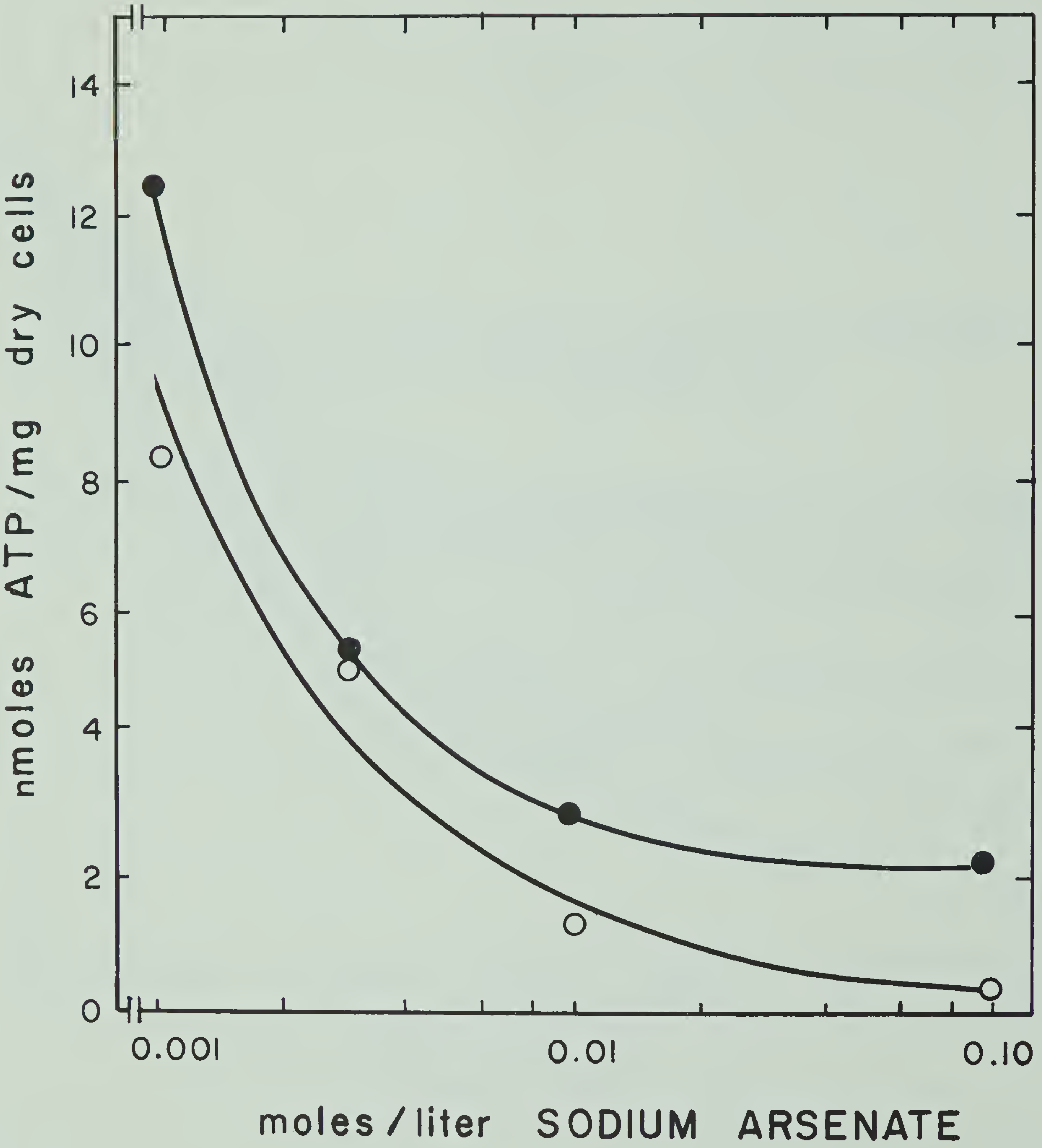

nmoles ATP/mg dry cells
0
2
4
6
8
10
12
14
0.001
0.01
0.10
moles/liter SODIUM ARSENATE

Figure 5.14. The effect of sodium arsenate on the ATP levels in *E. coli* K12

Data for this figure are compiled from those illustrated in Figures 5.12 and 5.13. Glycerol TMM culture (o); glucose TMM culture (●).

[illegible]

medium, although the effect is somewhat more marked in glycerol TMM than in glucose TMM-grown cells.

Figure 5.15 illustrates the relationship between per cent inhibition of uncoating and ATP levels in cells grown in the two media. In cells grown in glycerol TMM, there is a clear correlation between the per cent inhibition of uncoating and the decrease in ATP concentration produced by arsenate. The correlation is less clear in the case of glucose TMM-grown cells, in which uncoating was not markedly affected even in the face of an 80% reduction in cellular ATP.

These results are reminiscent of those obtained from the previously described experiments on the effects of anaerobiosis and DNP on uncoating. The implications of these findings will be discussed in the final section of this chapter.

### The effect of 2-deoxy-D-glucose on uncoating of phage R17 RNA by E. coli K12

Respiratory inhibitors and uncouplers operate on the late stages of carbohydrate metabolism, and the previous results have shown that they are able to effect a reduction in cellular ATP levels. In order to investigate an inhibitor that imposes a block at an earlier stage in the metabolic cycle of carbohydrates, the effects of 2-deoxy-D-glucose (2DG)

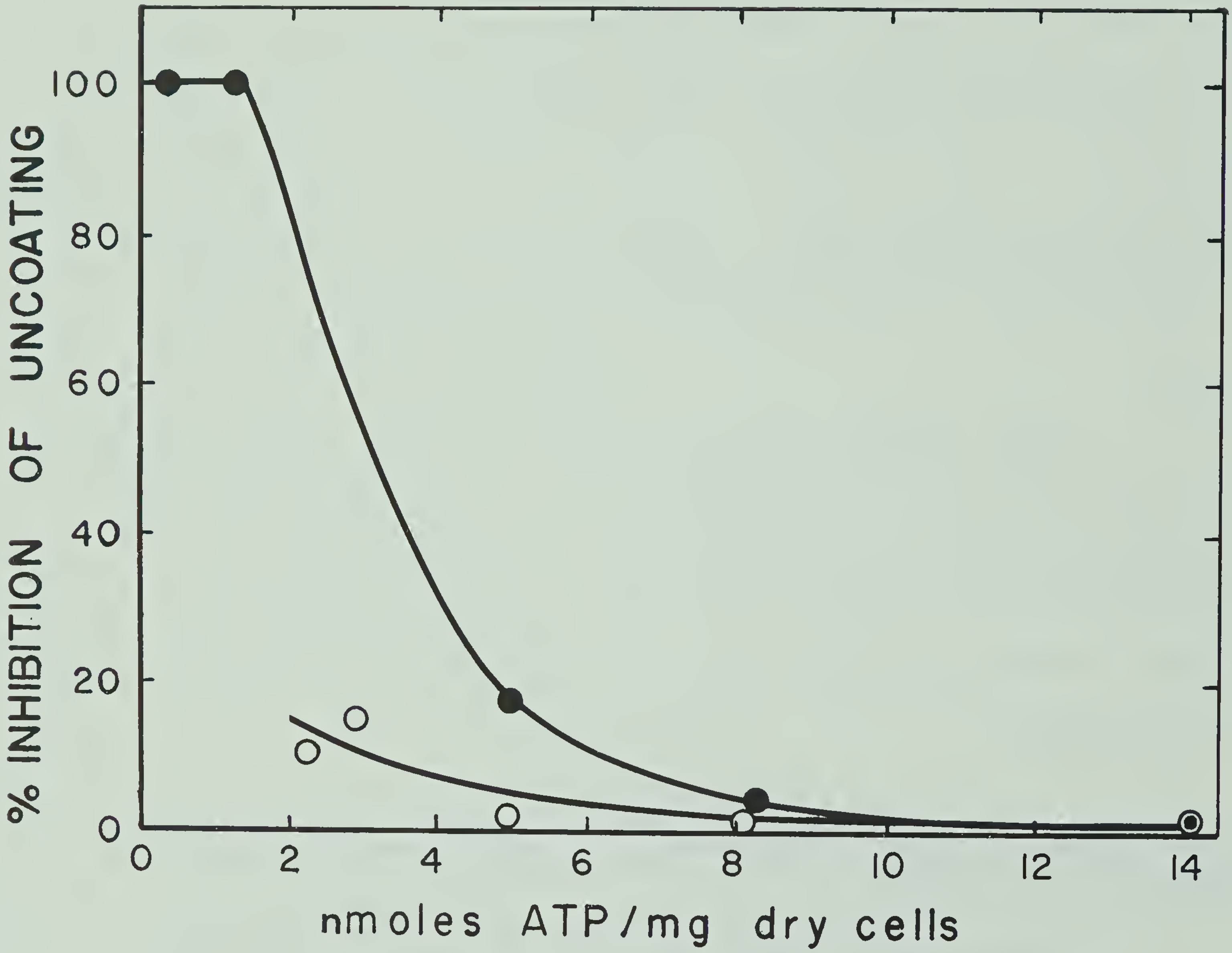

% INHIBITION OF UNCOATING
100
80
60
40
20
0
0
2
4
6
8
10
12
14
nmoles ATP/mg dry cells

Figure 5.15. The relationship between ATP levels and uncoating in arsenate-treated cultures of *E. coli* K12

Data for this figure were compiled from those illustrated in Figures 5.12 and 5.13. Glucose TMM culture (o); glycerol TMM culture (●).

on ATP levels in the uncoating of R17 phage by cells were examined. This compound is an inhibitor of glycolytic and oxidative conversion of glucose (Webb, 1966), and there are apparently several mechanisms by which 2DG produces an inhibition in the utilization of carbohydrate in mammalian organisms, molds, and tissue culture cells (Nirenberg and Hogg, 1958; Wick and Drury, 1957; Barban and Schulze, 1961). While the ability of *E. coli* B cells to take up 2DG has been questioned by Gershanovich (1963), we have found that 2DG causes inhibition of the uncoating reaction when glycerol is used as the carbon source in the culture. This inhibition is illustrated in more detail in Figure 5.16, from which it may be seen that concentrations of 500 μg./ml. and 1000 μg./ml. of 2DG caused a 20% inhibition of the uncoating capacity of the cells. That the *E. coli* K12 cells are probably permeable to 2DG is evidenced by the fact that this glucose analogue caused a 62% decrease in the ATP level of the culture. Neither inhibition of uncoating nor decrease in cellular ATP levels by 2DG was ever observed in cells grown in glucose. It is quite likely that when glucose is present in the culture medium, a small quantity of 2DG will not be taken up by the cells.

It is worth noting that increasing the concentration of 2DG from 500 μg./ml. to 1000 μg./ml. caused no

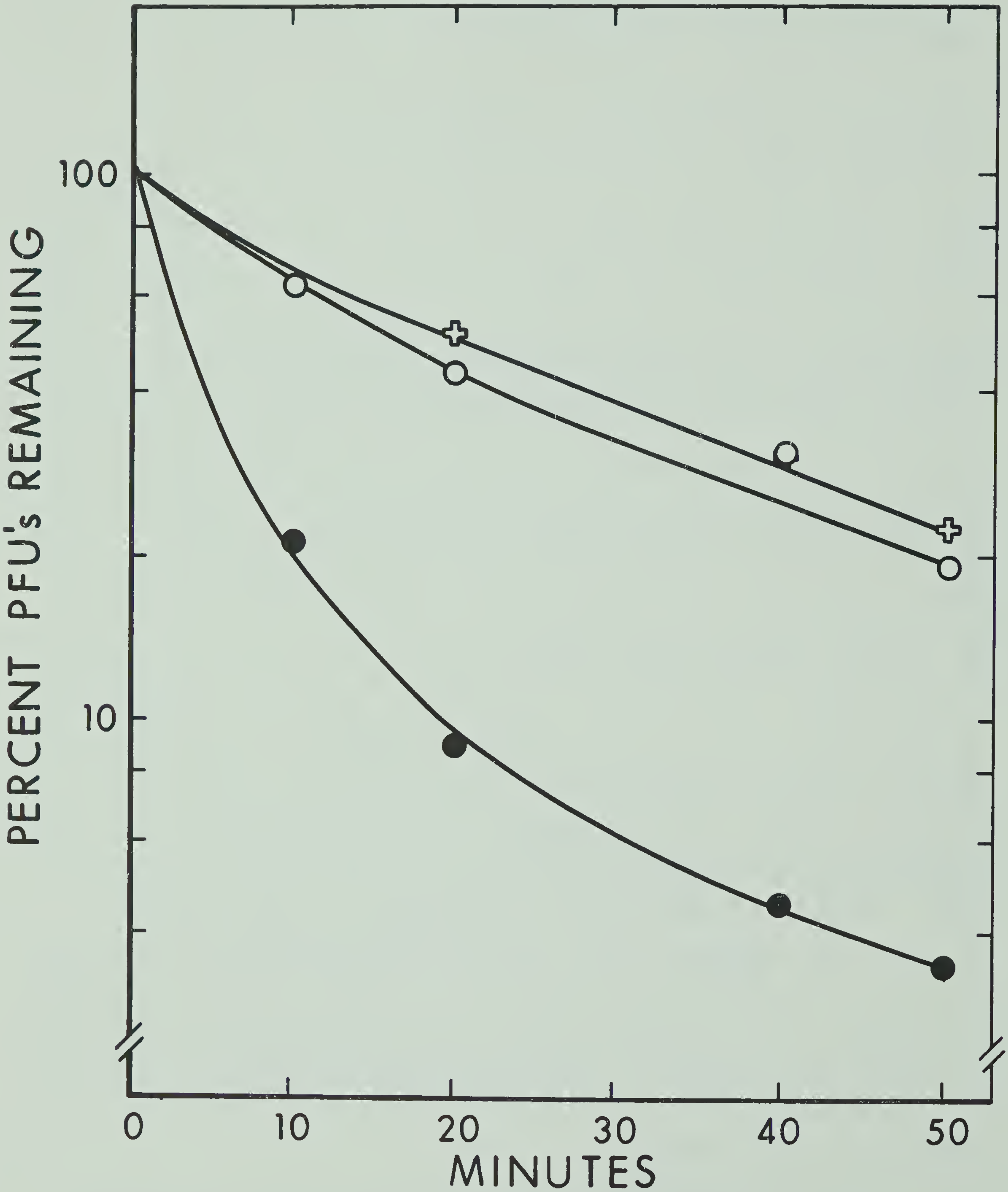

100
10
PERCENT PFU's REMAINING
0
10
20
30
40
50
MINUTES

Figure 5.16. The effect of 2-deoxy-D-glucose on the uncoating of R17 RNA by *E. coli* K12

A culture of *E. coli* K12 was grown in glycerol TMM to a density of 3 x $10^8$ bacteria/ml., after which RNase was added to give a final concentration of 100 μg./ml. Ten ml. aliquots were transferred to three separate flasks containing sufficient 2-deoxy-D-glucose to give the indicated concentrations, after which phage R17 was added to each flask at a multiplicity of 0.01 PFU/bacterium. The cultures were incubated at 37° and assayed for their capacity to uncoat phage RNA as described in Materials and Methods.

Control (no 2-deoxy-D-glucose) - 13.3 nmoles ATP/mg. (●);
500 μg./ml. 2-deoxy-D-glucose - 4.9 nmoles ATP/mg. (o);
1000 μg./ml. 2-deoxy-D-glucose - 5.0 nmoles ATP/mg. (+).

further decrease, either in the amount of uncoating or in the level of cellular ATP. This observation suggests that the inhibition of uncoating is not the result of a direct effect of the 2DG on the interaction of pili and phage, since, if the compound acted by this mechanism, one would expect the effect to be proportional to the amount of inhibitor over a greater concentration range.

### Uncoating of phage RNA by glucose-starved E. coli K12

When a culture of *E. coli* K12 is grown in limiting glucose medium (the limiting glucose medium contained methionine as the only amino acid, and the glucose concentration was 2.4 mM), a characteristic levelling off of cell growth is observed when the glucose in the culture medium is exhausted. If an uncoating experiment is carried out with such glucose-starved bacteria, the level of uncoating is found to be identical with that found in normal glucose TMM or glycerol TMM cultures. An observation which suggested that the energy required for the uncoating reaction may be supplied by a membrane-associated ATP, was that when glucose-starved cells were treated with Sm to block further pili growth (Brinton, 1965), and then supplied with glucose or its analog, 2DG, they lost some of their capacity to uncoat phage RNA (Figure 5.17). Figure 5.18 illustrates the effects of the above experimental conditions on cellular ATP levels.

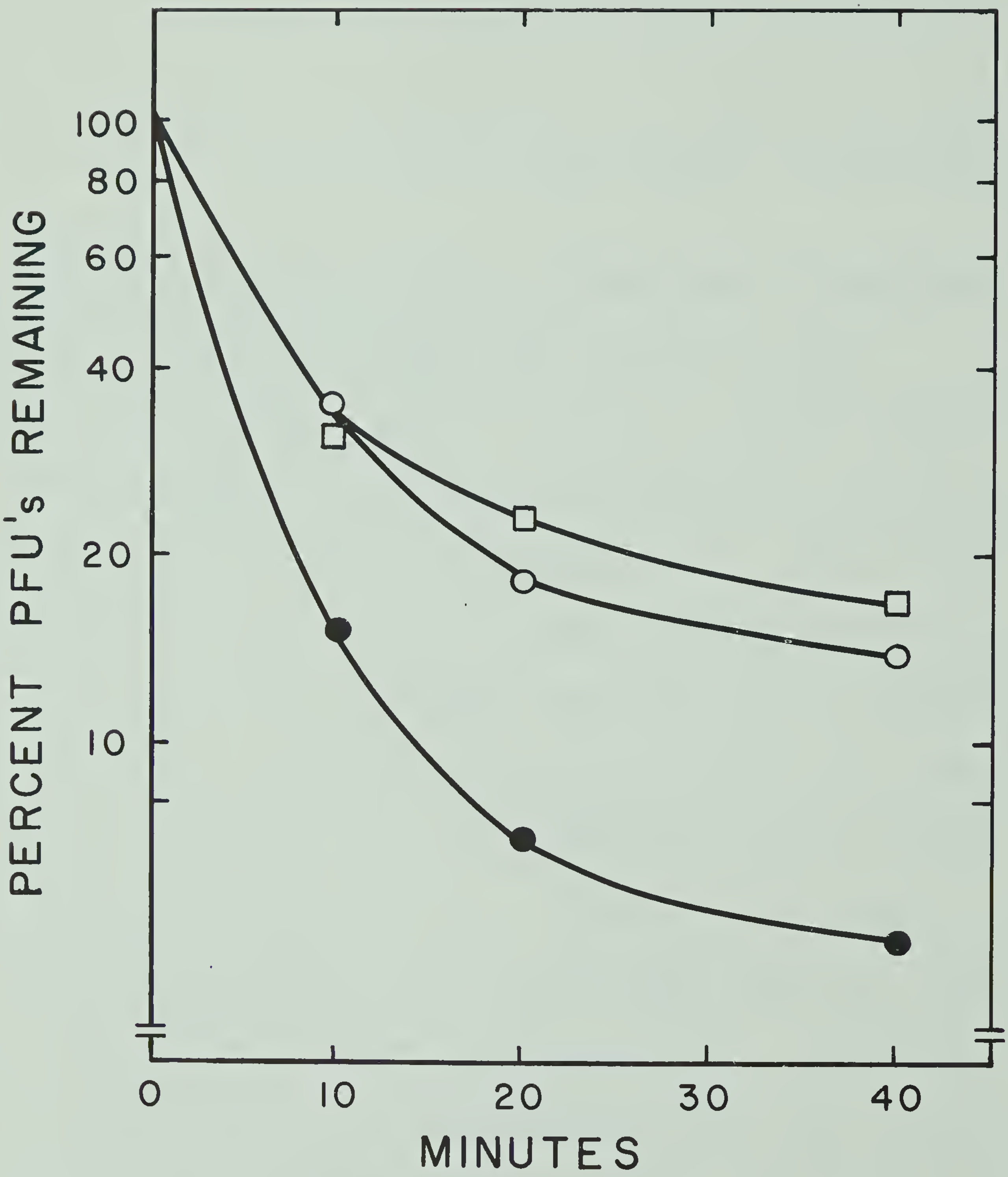

PERCENT PFU's REMAINING
100
80
60
40
20
10
0
10
20
30
40
MINUTES

Figure 5.17. The effect of glucose or 2-deoxy-D-glucose on the uncoating of R17 RNA by a glucose-starved culture of *E. coli* K12

A culture of *E. coli* K12 was grown in medium similar to glycerol TMM (the glycerol was replaced with 2.4 mM glucose) until the absorbance of the culture reached a constant level of approximately 0.55 $A_{650}$ units. After a further incubation of 60 minutes, the culture was made to contain 100 μg./ml. ribonuclease and 200 μg./ml. Streptomycin sulfate. The culture was incubated for an additional 15 minutes, and then it was divided into three portions. One portion served as a control, while the remaining two portions were made to contain glucose and 2-deoxy-D-glucose at a concentration of 3 mM. The cultures were incubated for 10 minutes, after which phage R17 was added at a multiplicity of 0.01 PFU's/cell and the cultures were assayed for uncoating activity as described in Materials and Methods. Control culture (●); glucose added (o); 2-deoxy-D-glucose added (□).

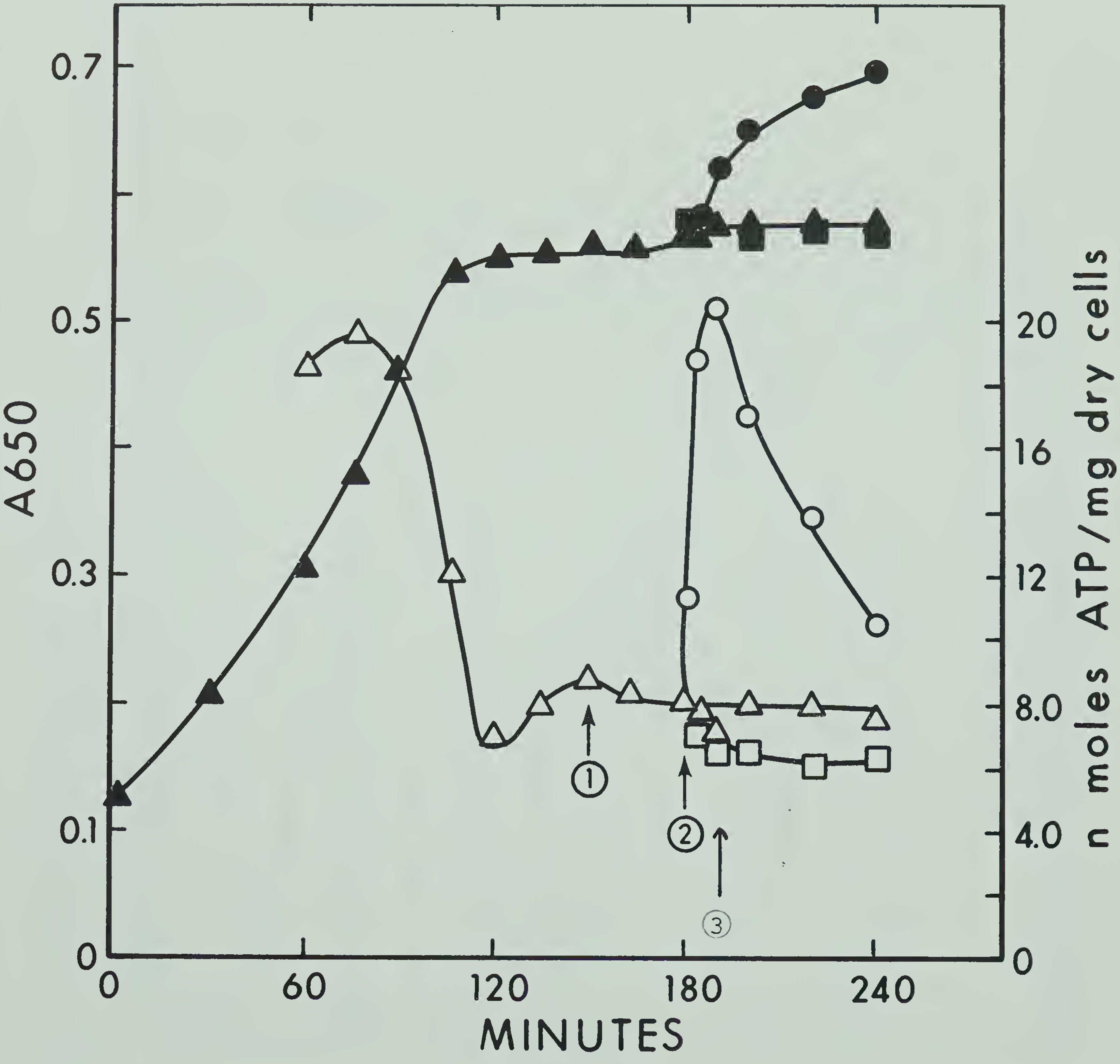

0.7
0.5
0.3
0.1
0
A650
0
60
120
180
240
MINUTES
20
16
12
8.0
4.0
0
n moles ATP/mg dry cells
①
②
③

Figure 5.18. The level of ATP in E. coli K12 during carbon starvation and replenishment

A culture of E. coli K12 was grown overnight in limiting glucose medium and diluted 1/100 into the same medium. Growth of the cells was followed by measuring optical density of the culture at 650 mμ (filled symbols). Samples were removed at intervals and assayed for ATP (open symbols) as described in the text. Thirty minutes after cessation of growth, Sm (100 μg./ml.) was added to the culture to prevent further pili growth (arrow ①). The culture was starved for an additional 30 minutes and then was divided into three equal aliquots (arrow ②). One was made 3 mM with respect to glucose (o), another was made 3 mM with respect to 2-deoxy-D-glucose (□), and the third was a control culture (△). ATP assays were carried out on all cultures simultaneously. Arrow ③ indicates the time at which sampling was started for the uncoating experiment shown in Figure 5.17.

It may be seen that depletion of the carbon (energy) source was followed by a rapid decrease in the level of ATP. The addition of glucose to the starved cells produced a rapid increase in ATP from about 6 to 18 nmoles/mg. dry weight, whereas the addition of 2DG produced a further slight decrease (6 to 5 nmoles/mg.).

Arrow 3 (Figure 5.18) indicates the time at which sampling for uncoating assays was started. It is clear that after the addition of either glucose or 2DG to the starved cells, there was a decrease in the ability of the cells to uncoat phage RNA.

Those results are compatable with the concept that glucose and 2DG are taken up by cells by means of an active transport mechanism that requires ATP, and that their uptake by starved cells lowers the level of membrane-associated ATP that may be required for the uncoating process.

### The effect of adenosine triphosphatases on uncoating of R17 RNA

Attempts to prevent the uncoating of bacteriophage RNA by pretreatment of bacterial cells with potato apyrase or actomyosin were unsuccessful. These results confirm the findings of Knolle (1967), who was unable to prevent RNA phage infection of *E. coli* with potato apyrase.

## Discussion

Although it seems most likely that phage attach to the F-pilus as the initial stage of a normal infection (possibly via interaction between the hydrophobic A protein of the phage and the lipoprotein of the F-pilus) (Roberts and Steitz, 1967), it is not clear what subsequent reactions must take place before the viral RNA reaches the host-cell ribosomes. Edgell and Ginoza (1965) have provided evidence that the coat protein remains outside the bacterium during infection; thus it is probable that the RNA and the protein coat are dissociated rather early in the infectious process. It can be demonstrated that the infectivity of R17 is completely resistant to 100 μg./ml. of pancreatic ribonuclease, an observation compatable with the hypothesis that RNA-capsid dissociation occurs early and at the surface of the F-pili.

The uncoating process is clearly an energy-requiring one, for which the calculated energy of activation is of the same order of magnitude as that for many biological processes.

The idea that the cell provides energy for the uncoating process is in agreement with the conclusions of Knolle (1967), who suggested that the invasion of host cell by the phage requires the active participation of the former. However, invasion, as defined by Knolle, would appear to mean the sum of all stages between adsorption and appearance

[illegible]

[illegible]

[illegible]

[illegible]

of progeny phage, whereas the uncoating assay developed by Paranchych (1966) provides a method for examining that stage of infection that occurs immediately after adsorption, and just prior to or coincident with, the penetration of the bacterium by RNA. Starving or prechilling bacterial cells inhibits invasion, as defined by Knolle, but has been shown to have no effect on their uncoating ability.

The effect on the uncoating reaction of a number of metabolic poisons, including respiratory inhibitors, uncouplers of oxidative phosphorylation, uncouplers of substrate-level phosphorylation and substrate analogues has been studied, and the results are summarized in Table II. All metabolic inhibitors studied caused the inhibition of phage uncoating when the cells were cultured in a glycerol TMM medium, and there was a good correlation between the degree to which uncoupling was inhibited and the extent to which the ATP content of the cells was decreased. The only instance in which there was any departure from this general rule was in the case of nitrogen, but it has been shown by Braak (1928), that anaerobiosis in glycerol cultures produces abnormal metabolic shifts which cause fermentation to cease before the glycerol is exhausted.

A correlation between inhibition of uncoating and decrease in cellular ATP levels was not found in the case of

## TABLE II

Relationship between inhibition of R17 RNA uncoating and overall levels of ATP in *E. coli* K12 treated with metabolic inhibitors

| Inhibitor | Concentration | Glycerol TMM | | Glucose TMM | |
|---|---|---|---|---|---|
| | | % Inhibition of Uncoating | [ATP] nmoles/mg. | % Inhibition of Uncoating | [ATP] nmoles/mg. |
| Control | - | 0 | 14.4 | 0 | 14.4 |
| Sodium arsenate | 1 x $10^{-3}$ M | 3 | 8.3 | 0 | 12.3 |
| Sodium arsenate | 3 x $10^{-3}$ M | 16 | 5.1 | 0 | 4.9 |
| Sodium arsenate | 1 x $10^{-2}$ M | 100 | 1.3 | 10 | 2.9 |
| Sodium arsenate | 1 x $10^{-1}$ M | 100 | 0.3 | 14 | 2.3 |
| Nitrogen | - | 95 | 7.2 | 14 | 7.1 |
| 2-Deoxy-D-glucose | 1 mg./ml. | 20 | 4.9 | 0 | 14.0 |

cells grown in glucose TMM medium. In such cells, no inhibitor examined inhibited uncoating by more than 14%, although several very effectively reduced ATP levels. This suggests that an alternate energy donor, either not made or made in small amounts in glycerol TMM, is produced in glucose TMM-grown cells, and that this compound can be utilized to provide the energy for the uncoating reaction.

Since uncoating takes place at the surface of the cell, it seems reasonable to suggest that a membrane-associated ATP may be involved in the phenomenon. It is, in fact, possible that membrane ATP is the primary energy source for this reaction, and that fluctuations in total cellular ATP produced by various inhibitors are merely fortuitous. This premise gains some credence from the observation that a small but reproducible inhibition of uncoating was produced in starved cells by the addition of glucose or 2DG. If these compounds are taken up by a process of active transport, perhaps involving a permease, as has been demonstrated for a number of carbohydrates in E. coli (Hagihira, et al., 1963), there may well be a requirement for phosphorylation of either the carbohydrates or their hypothetical "carrier protein" (Fox and Kennedy, 1967). The addition of glucose or 2DG to starved cells may, then, deplete the level of membrane-bound ATP, and thus inhibit the uncoating reaction. The data

arising from studies of the effects of ATPases make it clear that ATP is not situated on the outer surface of the membrane.

In summary, these studies have shown that uncoating is an energy requiring process. It is possible that one or more energy donors other than ATP can be utilized for this reaction, especially when glucose is present in the culture medium, although the nature of these compounds has not been specifically determined.

Even with the knowledge that energy is apparently required for phage uncoating to occur, it has not been possible to determine whether this is the sole reason for the inability of isolated F-pili to carry out the reaction, and the elucidation of the functional role of the pilus membrane (if it is a membrane) and the F-DNA will have to await further investigation.

# VI. THE FATE OF THE BACTERIOPHAGE COAT PROTEIN IN THE INFECTION OF *E. COLI* K12 WITH R17

## Introduction

RNA phage do not possess a tail structure, and thus, the means by which the RNA phage genome penetrates the bacterial cell is unclear. The discovery that F-pili are the adsorption sites for RNA phage did not completely resolve this question, although, a comparison of the width of the F-pilus with the diameter of the phage makes it difficult to imagine that the whole phage could enter the F-pilus. A study of this problem was undertaken by Edgell and Ginoza (1965), who examined the separation of coat protein and viral RNA, using doubly-labelled phage R17 after infection of a male strain of *E. coli* (HfrAB735HT). Their results suggested that only the phage RNA enters the bacterial cell, the protein coat remaining at the cell surface.

No further information regarding the fate of the nucleic acid-free coat protein has been obtained. It is not known whether the protein coat disintegrates completely into sub-units, or whether the viral RNA exits from the particle at a specific site leaving the capsid essentially intact. If the latter alternative were true, it would be of interest

to determine whether they remain attached to, or are released from, the F-pilus.

The investigations described in this chapter were designed to provide answers to these questions. Our approach was to infect *E. coli* K12 cells with phage labelled in both the protein and nucleic acid moieties, and then to determine the distribution of the protein label in sucrose gradients. Previous experiments had shown that RNase treatment of RNA phage gives rise to a large number of protein particles which have a sedimentation coefficient of approximately 42S, and which have the appearance of intact empty phage heads when examined under the electron microscope. Examination of the sucrose gradients of infected cultures suggested that R17 infection of *E. coli* K12 gives rise to analogous material which occurs free in the culture medium.

## Methods and Materials

### Bacteria

*E. coli* K12 was used throughout this study and was cultured in glucose TMM (described in Chapter II).

### Bacteriophage

The preparation of $C^{14}$-$H^{3}$-labelled phage was exactly as previously described for $P^{32}$-labelled phage (Chapter II),

except that $C^{14}$-uracil and $H^3$-lysine were used to label the nucleic acid and protein respectively. The isotopes were added to the culture at 10 minutes post-infection and the specific activity of the isolated phage was found to be 2.4 x $10^{-6}$ cpm/PFU and 8.8 x $10^{-7}$ cpm/PFU with respect to $H^3$ and $C^{14}$ respectively. Thus, the ratio of $H^3$ counts/$C^{14}$ counts for the intact purified phage was 1.6. The particle/PFU ratio, although not directly measurable because of the small amount of phage prepared, was probably close to 10, the ratio of total to infective particles usually found in most of our phage preparations. This was the value prior to prolonged RNase treatment.

## Sucrose gradients

Linear, 5 - 20% sucrose gradients were prepared in either Buffer A (0.1 M Tris, pH 7.4) or Buffer B (0.01 M Tris, 1 mM $MgCl_2$, 40 mM KCl, pH 7.8). Gradients were allowed to equilibrate at 4° overnight before sample application. Sample volumes were 0.2 ml., gradient volumes were 4.8 ml., and the rotor used was the SW39. Twenty-four fractions of 12 drops each were collected from each gradient by puncturing the bottom of the cellulose nitrate rotor tube, using a specially designed hollow needle assembly (obtained from Buchler Instruments).

## Radioactive measurements

Radioactivity of phage samples was determined using a Beckman LS200 liquid scintillation spectrometer. In double labelling experiments, the $C^{14}(-H^3)$ module was used in conjunction with the variable voltage window which was set at 0 - 0.9 volts. The overflow of $C^{14}$ into the tritium channel was adjusted to a constant value of 10%. Brays dioxane base scintillation fluid (Bray, 1960) was used in place of the previously described toluene scintillation fluid because of the immiscibility of the gradient fractions in the toluene. All counting which involved the determination of the ratio of coat protein to nucleic acid radioactivity was carried out for a sufficiently long period of time to insure that the counting error was not greater than 5%.

## Ribosomal sub-units

Fifty and 30S sub-units of purified *E. coli* B ribosomes, which were used as markers in the gradients, were the gift of Dr. S. Igarashi.

## Results

### The ribonuclease sensitivity of RNA phage

It is commonly accepted knowledge that Ribonuclease A treatment of RNA phage preparations does not result in a

significant loss of infectivity. However, when $H^3C^{14}$-labelled phage R17 (purified according to the normal procedures and subjected to an additional purification in a sucrose gradient) was treated with RNase (100 μg./ml.) for 30 minutes at 37°, the phage population was resolved into two peaks of radioactivity when analyzed in sucrose gradients (Figure 6.1), indicating that many of the non-infectious particles in the preparation were susceptible to RNase. The details of this experiment were as follows. An aliquot of the RNase-treated phage stock was layered on a linear 5 - 20% sucrose gradient in Buffer B. Fifty and 30S sub-units of *E. coli* B ribosomes were placed on an identical gradient (Buffer B was used in this instance since, in the absence of $MgCl_2$ and KCl, ribosomal sub-units do not show characteristic sedimentation properties), and both gradients were centrifuged in the same SW39 rotor for 115 minutes at 35,000 rpm in a Spinco Model L preparative ultracentrifuge. Fractions were then collected as described in the Materials and Methods section of this chapter. The ribosomal material was non-radioactive, and its position was monitored by measurement of the 260 mμ absorbancy in a Zeiss spectrophotometer. The phage samples were assayed for radioactivity according to the normal procedure, and the two gradient profiles are super-imposed in Figure 6.1. It may be seen that

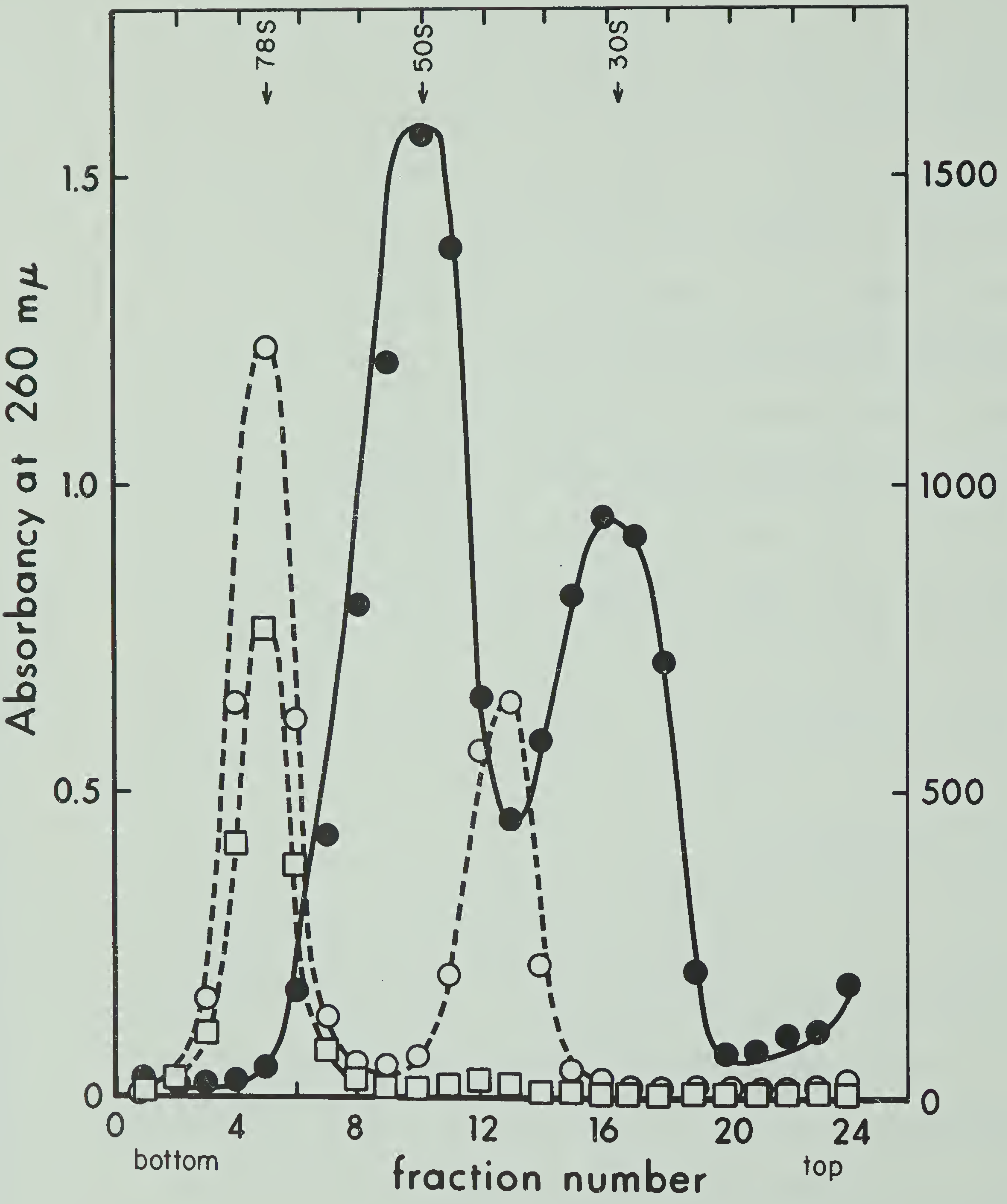

78S
50S
30S
Absorbancy at 260 mμ
1.5
1.0
0.5
0
counts per minute
1500
1000
500
0
0
4
8
12
16
20
24
bottom
fraction number
top

Figure 6.1. Sucrose gradient sedimentation analysis of nucleic acid-free phage protein produced upon RNase treatment of intact phage

Details of this experiment are described in the text. Subunits of *E. coli* ribosomes having a sedimentation coefficient of 30S and 50S were used as markers. They were centrifuged in an identical but separate gradient. Both gradients were centrifuged in a single centrifugation. Absorbancy at 260 mμ (——●——); H$^3$ (---o---); C$^{14}$ (---□---).

the major peak of radioactivity, corresponding to intact phage, contained both the $H^3$ and $C^{14}$ isotopes and sedimented with the expected sedimentation value of 78S. In addition to this material, there was a second peak of radioactivity which contained only tritium (the isotope used to label the coat protein). This fraction sedimented at a position in the sucrose gradient between the 50S and 30S ribosomal sub-units. Using the relationship established by Martin and Ames (1961), that in a linear gradient, molecules move a distance from the meniscus which is directly proportional to their S value, the sedimentation coefficient of this tritium peak was calculated to be approximately 42S.

It is obvious from the sedimentation characteristics of the 42S fraction, that it is not composed of dissociated coat protein sub-units, since such molecules would have a molecular weight of 14,200, and would therefore remain close to the top of the gradient under the conditions used.

The two separated peaks were dialyzed against Buffer A to remove sucrose, stained with phosphotungstic acid (PTA), and examined under the electron microscope to determine whether the material was structurally identifiable. Plate III shows three electron micrographs of RNase-treated phage which illustrates our findings. The large micrograph shows the phage stock before centrifugation, while the small

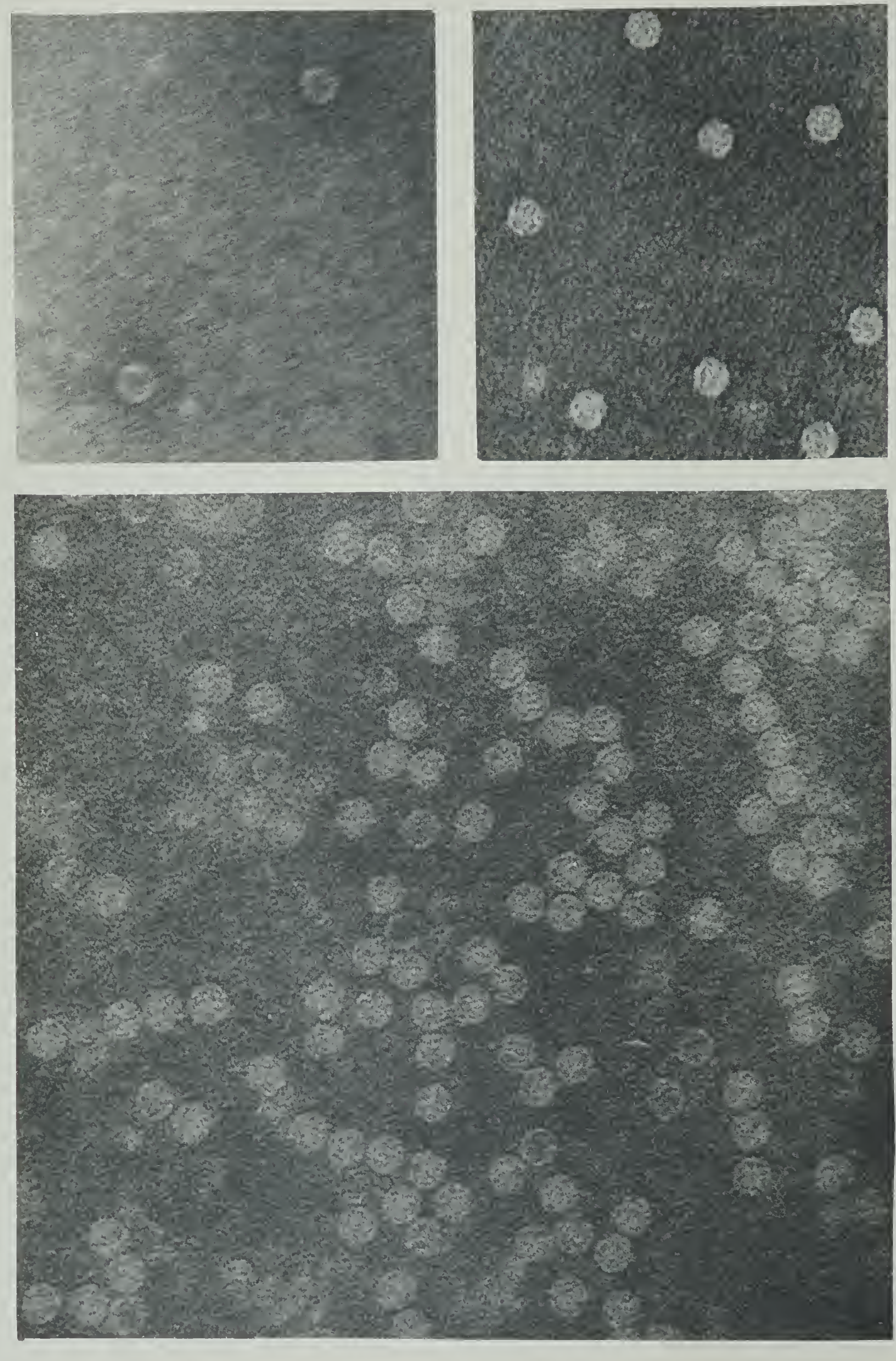


Plate III. Electron micrographs of RNase-treated phage R17

Bacteriophage R17, which had been purified by normal procedures and which had received an additional purification in a sucrose gradient, were treated for 30 minutes at 37° with 100 μg./ml. Ribonuclease A. The treated phage were analyzed in a sucrose gradient as described in the text. The peak fractions of the gradient were dialyzed versus Buffer A to remove sucrose, stained with PTA and examined under the Phillips 200 electron microscope. Upper left - phage particles from region of sucrose gradient having sedimentation coefficient of 42S; upper right - phage particles from region of sucrose gradient having sedimentation coefficient of 78S; lower - phage particles from unfractionated phage preparation.

micrographs show particles from the isolated peaks. The low concentration of particles in the small micrographs reflects the low phage concentration in the fractions recovered from the gradient.

The large micrograph resembles a typical phage population (c.f. Plate I), with the exception that there are apparently phage particles with light cores and a small fraction with dark cores. The significance of the dark and light cores of these particles can only be made clear when the mechanism of PTA staining of the phage is understood. If nucleic acid is present within a phage particle, the PTA stain (which prevents the transmission of electrons) is excluded from the core of the particles. Electrons pass through the phage, however, impinging upon the film beneath, and causing it to darken. When a positive print of the film is made, the dark and light areas are reversed, and the central core appears as a light area. If the particle is empty, PTA can diffuse in, and by similar reasoning the core appears dark on the printed micrograph.

It may be seen that the unfractionated phage preparation contained mainly intact phage particles, with the occasional "empty" particle also being visible. The phage particles from the 78S region of the sucrose gradient (upper right plate) consisted almost entirely of complete particles,

while the phage particles from the 42S region of the sucrose gradient (upper left plate) contained only "empty" particles. These visual observations agree well with the observed radioactivity of these preparations, i.e. $C^{14} + H^{3}$ in the 78S region and $H^{3}$ alone in the 42S peak. The experiment therefore indicates that RNase treatment converts a fraction of intact phage to "empty" particles. Furthermore, these nucleic acid-free particles can be separated from intact phage particles in a sucrose gradient, and they sediment with an S value of approximately 42S.

In light of the resistance of phage infectivity to RNase, these findings suggest that there are a large number of particles produced in R17-infected cells which are non-infectious, although they contain RNA and behave as normal phage in all the purification procedures. These phage could possibly differ from normal R17 by not containing the A protein sub-unit, in which case, they would be non-infectious because of an inability to adsorb to F-pili, and their nucleic acid content would be accessible to RNase if the absence of the A protein caused the phage particle to be permeable to RNase. Apparently the brief RNase treatment used in the initial purification of the phage to remove ribosomal material is not sufficient to degrade all these abnormal particles.

## The fate of the bacteriophage coat protein during the infection of *E. coli* K12 with R17

The production of phage ghosts by RNase suggests that the normal release of R17 RNA during infection of *E. coli* could come about in the following manner. Following phage attachment to an F-pilus by means of the A protein subunit, an energy-requiring event results in the dissociation of the A protein from the phage particle. This dissociation of the A protein from the phage presumably provides an opening through which the phage RNA is released either into or onto the F-pilus, leaving behind an "empty" phage particle. The "empty" phage particle would then be released from the pilus since it no longer contains its attachment site (A protein).

This hypothesis predicts that it should be possible to find "empty" phage particles (similar to those produced by RNase treatment) in a bacterial culture after infection with intact phage. Such an experiment was, in fact, carried out as follows. A culture of *E. coli* K12 was grown to a density of $6 \times 10^8$ bacteria per ml. and was then infected at a multiplicity of 30 PFU/bacterium with doubly labelled phage R17 (the phage had been purified, RNase treated, then repurified on a sucrose gradient to remove RNase sensitive particles). The culture was incubated at $37^o$ for 30 minutes,

and then chilled. One-half the culture was untreated, while one-half was vigorously blended with glass beads in a Vortex mixer to remove F-pili from the cells. A portion of each culture was then layered onto each of two sucrose gradients (5 - 20% in Buffer A) and the gradients were centrifuged for 115 minutes at 35,000 rpm. The fractions were collected and assayed as previously described, and the results of the experiment are illustrated in Figures 6.2 and 6.3. Two peaks were found in a sucrose gradient analysis of the infected, unblended culture (Figure 6.2), one corresponding to intact phage, and the other to phage ghosts. The same two peaks were seen in a sucrose gradient analysis of the blended culture (Figure 6.3). Although the size of the main phage peak was somewhat larger in the case of the blended culture, the size of the "ghost" peak was about the same in both.

Unfortunately, the radioactivity of the 42S peaks was very low, which made it meaningless to analyze the data in strictly quantitative terms. However, a semi-quantitative analysis indicated that approximately 4% of the input phage particles lost their RNA. Table III shows the distribution of radioactivity in the two sucrose gradients illustrated in Figures 6.2 and 6.3. In the unblended culture, 3.2% of the total input phage coat protein appeared in the 42S region of the gradient, while 4.5% of the input phage coat protein

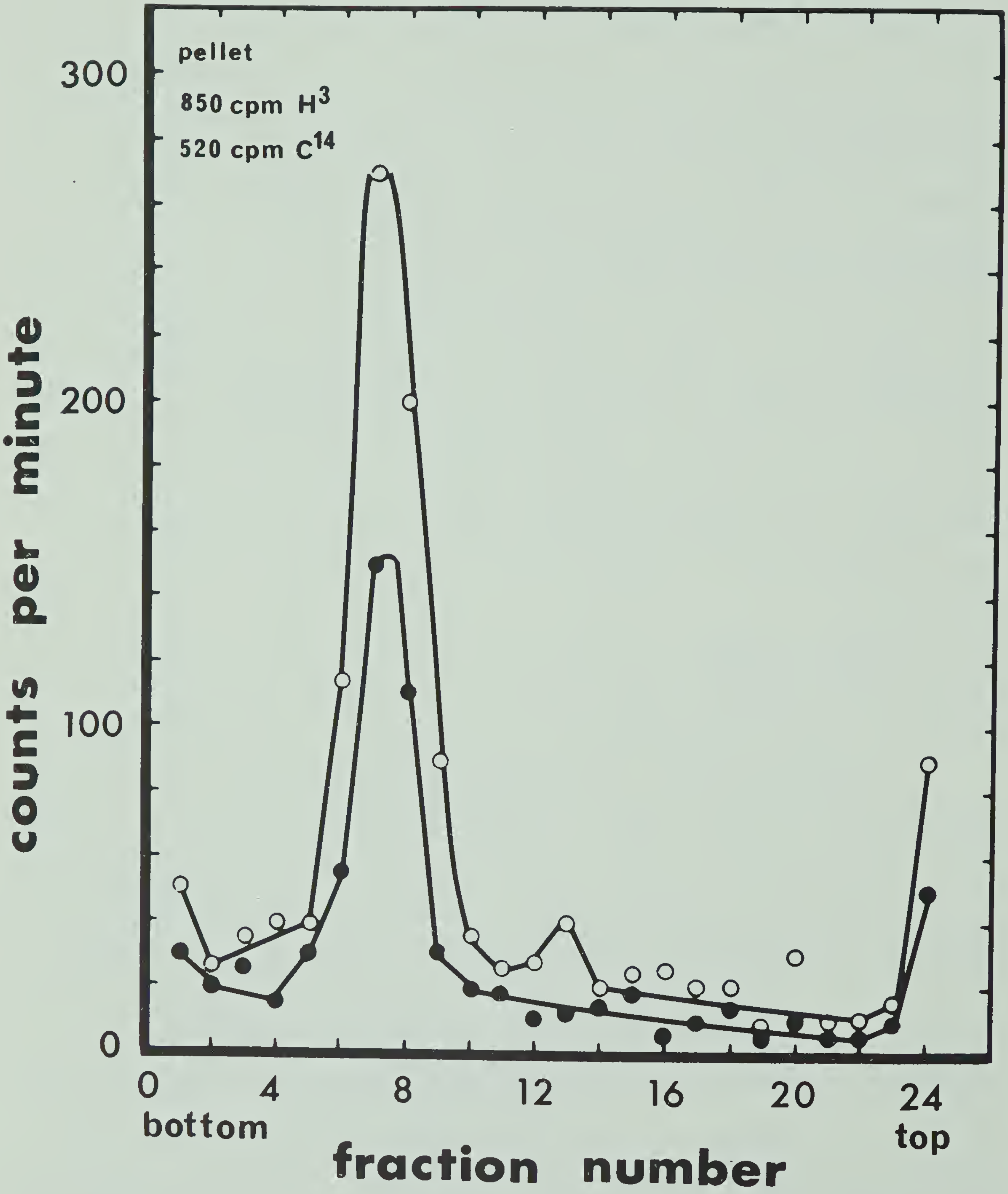

pellet
850 cpm $H^3$
520 cpm $C^{14}$
counts per minute
300
200
100
0
0
4
8
12
16
20
24
bottom
top
fraction number

Figure 6.2. Sucrose gradient profile of an R17-infected culture of *E. coli* K12

A culture of *E. coli* K12 was grown with slow shaking to a density of 6 x $10^8$ cells per ml. $H^3$-$C^{14}$-labelled R17 was added at a multiplicity of 30 PFU's/bacterium and infection was allowed to proceed for 30 minutes at 37°. The culture was subsequently chilled, and an aliquot of 0.2 ml. was layered on a linear 5 - 20% sucrose gradient in Buffer A. After 115 minutes of centrifugation at 35,000 rpm, fractions were collected as previously described in the text, and they were monitored for radioactivity. $H^3$ (o); $C^{14}$ (●).

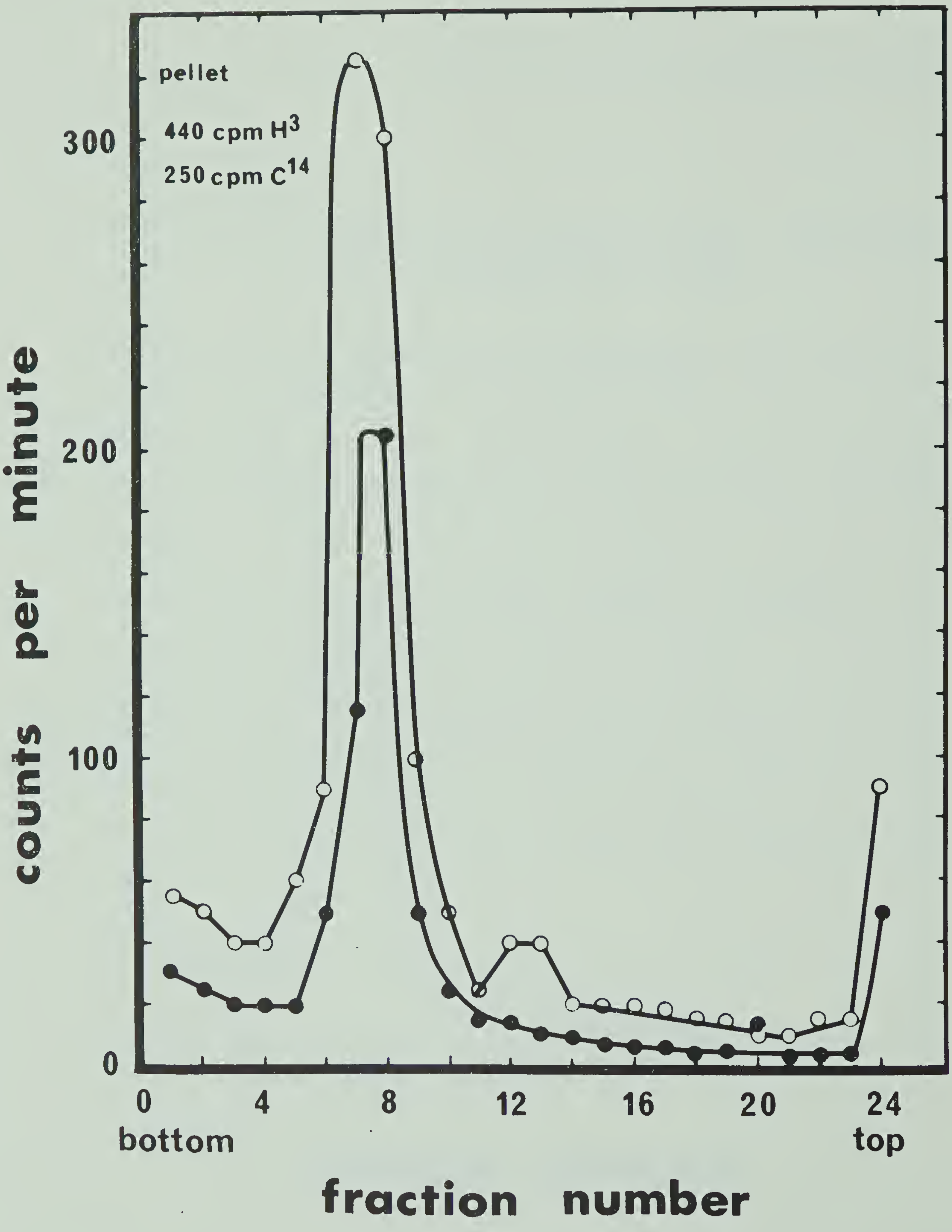

pellet
440 cpm $H^3$
250 cpm $C^{14}$
300
200
100
0
counts per minute
0
4
8
12
16
20
24
bottom
top
fraction number

*Figure 6.3*. Sucrose gradient profile on an R17-infected culture of *E. coli* K12 after blending

Details for this experiment are as described in the legend of Figure 6.2. The chilled infected culture was, however, blended in a Vortex mixer with glass beads before an aliquot of 0.2 ml. was layered on the gradient. $H^3$ (o); $C^{14}$ (●).

## TABLE III

Distribution of radioactivity in sucrose gradients of $H^3$-$C^{14}$-labelled R17-infected cultures of *E. coli* K12

Blended Culture

| | pellet cpm | 78S region cpm | 42S region cpm | meniscus cpm |
|---|---|---|---|---|
| $H^3$ | 440 | 885 | 80 | 90 |
| $C^{14}$ | 250 | 490 | 25 | 50 |
| $H^3/C^{14}$ | 1.8 | 1.8 | 3.2 | 1.8 |

Total $H^3$ = 1903 cpm Total $C^{14}$ = 988 cpm

Unblended Culture

| | pellet cpm | 78S region cpm | 42S region cpm | meniscus cpm |
|---|---|---|---|---|
| $H^3$ | 850 | 775 | 67 | 90 |
| $C^{14}$ | 520 | 350 | 15 | 45 |
| $H^3/C^{14}$ | 1.6 | 2.2 | 4.5 | 2.0 |

Total $H^3$ = 2055 cpm Total $C^{14}$ = 1148 cpm

| | Input phage (cpm) |
|---|---|
| $H^3$ | 2532 |
| $C^{14}$ | 1547 |
| $H^3/C^{14}$ | 1.64 |

from the blended culture sedimented at this position. This region of the gradient contained a significant amount of excess tritium (the average $H^3/C^{14}$ ratio was 3.7 compared with 1.6 for the input phage), which is in agreement with the previous data that showed the 42S peak of RNase-treated phage to consist primarily of empty $H^3$-labelled phage particles which were free of $C^{14}$ (RNA). It is likely, therefore, that there has been an average conversion of approximately 3 - 4% of the phage into empty particles as a result of the normal infection. Interestingly enough, this percentage is what one would theoretically expect on the following basis. According to the published distribution of F-pili in cultures of E. coli K12 (Brinton, 1965), approximately 30% of the bacteria have a single F-pilus and 60% have none. Therefore, out of $6 \times 10^8$ cells, only $2.4 \times 10^8$ F-pili should be available for uncoating phage. Furthermore, it is not likely that each pilus is capable of causing the uncoating of more than a few (2 - 5) phage particles because of an exclusion mechanism (Brinton and Beer, 1967). It follows that the maximum number of phage particles which could lose their RNA complement in this experiment as a result of phage-pili interaction, would be $1.2 \times 10^9$ out of $1.8 \times 10^{10}$ (input phage) or approximately 6 - 7% of the total. The 3 - 4% of $H^3$

radioactivity found in the 42S fractions of the blended and unblended cultures thus agrees well with these expectations.

It is to be noted that the ratio of $H^3$ counts/$C^{14}$ counts in the pellet, 78S, and meniscus fractions, is somewhat higher than that of the input phage. This most probably reflects the lack of precision of the measurements, although it is also possible that some "empty" or partially empty phage particles were present as contaminants in the pellet and 78S fractions. The significance of the small amount of radioactivity found at the miniscus is not presently understood. The radioactivity could be due to phage degradation products arising from the complete disruption of phage particles into sub-units or, alternatively, it may represent radioactivity which was washed from the pellet as the final portion of the gradient dripped through the bottom of the centrifuge tube.

It is therefore not possible at this time to state unequivocally that all phage particles which undergo uncoating remain as intact empty shells. It is probable, however, that a considerable portion of uncoated particles do so. Moreover, the observation that the amount of material in the 42S fraction did not increase significantly after subjection of the culture to a blending treatment, indicates

that the uncoated phage particles may become detached from the F-pilus following the release of phage RNA.

## Discussion

It is clear that RNase treatment of phage, labelled both in the nucleic acid and coat protein, releases RNA from a certain per cent of the population and thereby produces empty viral capsids which have a sedimentation coefficient of approximately 42S.

These non-infectious RNase-sensitive phage particles manifest the same sedimentation characteristics as normal infectious phage, since they were not separable from normal particles during the purification procedure. In addition, preparations of phage R17 also contain particles (detected during CsCl density gradient centrifugation) which have a buoyant density somewhat less than that of normal phage. These phage are also non-infectious, apparently because the particles lack a portion of their RNA.

It is certain that the spectrum of particle types in the normal phage population encompasses numerous other variations. It is therefore important that the purification of phage for experiments of the type described in this chapter, succeed in removing as many of these non-infectious particles as possible. This is why multiple gradient

centrifugation, followed by extensive RNase treatment and additional gradient purification, was used to prepare our input phage.

The initial results on the fate of the coat protein during infection are of sufficient interest to warrant further investigation. The phage stock used in these experiments was of a low specific activity because of a lack of sufficient label. Ideally, however, this experiment should be repeated with a phage stock which has been heavily labelled, both in the coat protein and in the nucleic acid. The use of such phage of high specific activity would allow a more accurate and quantitative treatment of the data. Moreover, it would allow a more unequivocal conclusion to be drawn regarding the question of whether phage ghosts become detached from pili upon release of phage RNA.

A second problem that should be pursued is the determination of the function of the A protein in infection. Specifically, if this molecule represents the adsorption site on the phage, it should be possible to detect A protein binding to F-pili. This is possible, theoretically, since the amino acid histidine is absent from all the coat protein sub-units, but is present in the A protein (Nathans and Oeschger, 1966). Growth of phage in the presence of radioactive histidine should thus yield phage labelled in the A

protein, but unlabelled in other coat components. The histidine-labelled phage could then be added to a culture to determine whether the histidine remains bound to the main coat protein component of the phage particle after infection, or whether it remains bound to the F-pilus and separates from the empty protein shell.

# SUMMARY

1. A filtration assay was established to measure the binding of RNA bacteriophage to F-pili. The assay involved the use of cellulose acetate filters which permitted the passage of free phage to the extent of 99.5%, but which retained phage-pili complexes.

2. Mono- and divalent cations were found to promote RNA phage binding to F-pili in increasing amounts strictly as a function of the ionic strength of the incubation mixture. However, non-electrolytes, as represented by sucrose, were ineffective in promoting phage adsorption.

3. The association state of F-pili was examined as a function of the salt concentration of the suspending medium by sucrose density gradient analysis. The addition of various salts to sucrose gradients of F-pili did not cause a change in the sedimentation pattern as would have been expected if the pili either associated or dissociated. Salt induced changes in the aggregation state of F-pili were thus ruled out as possible sources of error in the filtration assay method.

4. The effect of various shearing forces and culture conditions were examined to determine their effect on the

proportion of F-pili that remained associated with bacteria. It was found that the passage of cells through a narrow gauge needle, or the resuspension of cells by pipetting, provided sufficient shearing forces to cause substantial depiliation of bacteria. In addition, vigorous aeration of a growing culture of *E. coli* K12 was found to completely remove F-pili with 15 minutes.

5. In cultures grown under conditions where there was minimal breakage of F-pili, the amount of F-pili which occurred free in the supernatant was only 10% of the total F-pili produced by the cells during the same time period. This observation supports the hypothesis of "limited growth" in which F-pili remain associated with the bacterial cell following completion of growth.

6. At concentrations of salt which gave the optimal binding of phage to F-pili, the phage were found to be weakly bound with an equilibrium constant of $2.0 \times 10^{-4}$ ml./cpm.

7. When cultures were grown in glycerol TMM, the bacteria-mediated release of RNA from the bacteriophage was shown to be inhibited by various metabolic poisons or inhibitors such as nitrogen, 2,4-dinitrophenol, sodium arsenate, and 2-deoxy-D-glucose. Using a sensitive method for the

detection of ATP, which measured quantities as low as 1 μμmole, it was found that ATP levels were decreased in glycerol cultures under the same conditions that produced inhibition of uncoating. When glucose was used as the carbon source in the culture, there was only a negligible inhibition of uncoating, although ATP levels were decreased by as much as 90%. This latter observation was taken to mean that an energy source other than ATP is produced in glucose-grown cells, and that this energy source is capable of causing uncoating of phage RNA.

When glucose or 2-deoxy-D-glucose was added to a culture which had been starved of glucose and non-essential amino acids, the cells showed a diminished ability to uncoat phage RNA. ATP levels rose in the culture with the addition of glucose, while the addition of 2DG caused a slight decrease. The inhibition of uncoating which occurred in both these cases was attributed to competition between phage and sugar molecules for a limited supply of a membrane-associated energy donor.

8. RNase treatment of a purified preparation of phage R17 caused the conversion of a portion of the particles

into phage ghosts which sedimented with a sedimentation coefficient of approximately 42S. When phage, which had been repurified to remove all 42S material, was added to a culture of _E_. _coli_ K12 and the culture was analyzed on a sucrose density gradient, a small peak was again observed at the 42S region. This observation indicates that the F-pilus-induced release of RNA from the phage particle probably does not result in the concomitant degradation of the capsid into capsomeres. In addition, evidence is present which suggests that the empty capsids do not remain attached to the F-pilus.

# BIBLIOGRAPHY

Anderson, T.F. 1949. The Nature of the Bacterial Surface, Blackwell Scientific Publications, Oxford, 40.

Barban, S. and H.O. Schulze. 1961. J. Biol. Chem. 236, 1887.

Beutler, E. and M.C. Baluda. 1964. Blood 23, 688.

Bishop, D.H.L. and D.E. Bradley. 1965. Biochem. J. 95, 82.

Braak, H.R. 1928. Dissertation, Delft.

Bradley, D.E. 1966. 6th Int. Cong. for Electron Microscopy (Kyoto), 137.

Bray, B.A. 1960. Anal. Biochem. 1, 279.

Brinton, C.C. Jr. 1959. Nature 183, 782.

Brinton, C.C. Jr., P. Gemski Jr., S. Falkow and L.S. Baron. 1961. Biochem. Biophys. Res. Comm. 5, 293.

Brinton, C.C. Jr., P. Gemski Jr. and J. Carnahan. 1964. Proc. Natl. Acad. Sci. U.S. 52, 776.

Brinton, C.C. Jr. 1965. Trans. N.Y. Acad. Sci. 27, 1003.

Brinton, C.C. Jr. and H. Beer. 1967. The Molecular Biology of Viruses, (Colter and Paranchych, eds.), Academic Press, N.Y.

Caro, L. and M. Schnös. 1966. Proc. Natl. Acad. Sci. U.S. 56, 126.

Cole, H.A., J.W.T. Wimpenny and D.E. Hughes. 1967. Biochim. Biophys. Acta. 143, 445.

Crane, R.K. and F. Lipmann. 1953. J. Biol. Chem. 201, 235.

Crawford, E.M. and R.F. Gesteland. 1964. Virology 22, 165.

Davern, C.I. 1964. Australian J. Biol. Sci. 17, 719.

Davis, J., J. Strauss and R.L. Sinsheimer. 1961. Science 134, 1427.

Davis, J.E. and R.L. Sinsheimer. 1963. J. Mol. Biol. 6, 203.

Dubin, D.T., R. Hancock and B. Davis. 1963. Biochim. Biophys. Acta. 74, 476.

Duguid, J.P., I.W. Smith, G. Dempster and P.N. Edmunds. 1955. J. Pathol. Bacteriol. 70, 335.

Ellis, D.B. and W. Paranchych. 1963. J. Cell. Comp. Physiol. 62, 207.

Enger, M.D., E.A. Stubbs, S. Mitra and P. Kaesberg. 1963. Biochemistry U.S.A. 19, 857.

Feary, T.W., E. Fisher and T.N. Fisher. 1964. J. Bacteriol. 87, 196.

Fouace, J. and J. Huppert. 1962. J. Compt. Rend. 254, 4387.

Fox, C.F., J.R. Carter and E.P. Kennedy. 1967. Proc. Natl. Acad. Sci. 57, 698.

Gemski, P. Jr. 1964. Ph.D. Thesis, University of Pittsburgh.

Gershanovich, V.N. 1962. Biochemistry U.S.S.R. (English translation) 27, 868.

Gesteland, R.F. and H. Boedtker. 1964. J. Mol. Biol. 8, 496.

Green, H. and O. Meyerhof. 1952. J. Biol. Chem. 197, 347.

Hagihira, H., T.H. Wilson and E.C.C. Lin. 1963. Biochim. Biophys. Acta. 78, 505.

Hershey, A.D. and M. Chase. 1952. J. Gen. Physiol. 36, 39.

Hofschneider, P.H. 1963. Z. Naturforschung, 18b, 203.

Houwink, A.L. and W. van Iterson. 1950. Biochim. Biophys. Acta. 5, 10.

Hudson, J.B. 1967. Ph.D. Thesis, University of Alberta, Edmonton.

Huennekens, F.M. and H.R. Whiteley. 1960. Comprehensive Biochemistry, (Florkin and Mason, eds.), Academic Press, N.Y., 107.

Ippen, K.A. and R.C. Valentine. 1967. Biochem. Biophys. Res. Comm. 27, 674.

Ishibashi, M. 1967. J. Bacteriol. 93, 379.

Knolle, P. and F. Kaudewitz. 1962. Biochem. Biophys. Res. Comm. 9, 208.

Knolle, P. 1967a. Zentralbl. Bakteriol. I. Abtlg. Orig. 202, 33.

Knolle, P. 1967b. Zentralbl. Bakteriol. I. Abtlg. Orig. 202, 40.

Knolle, P. 1967c. Zentralbl. Bakteriol. I. Abtlg. Orig. 202, 417.

Kozloff, L.M. and M. Lute. 1959. J. Biol. Chem. 234, 539.

Lodish, H.F., K. Horiuchi and N.D. Zinder. 1965. Virology 27, 139.

Lodish, H.F. and N.D. Zinder. 1965. Biochem. Biophys. Res. Comm. 19, 269.

Loeb, T. and N.D. Zinder. 1961. Proc. Natl. Acad. Sci. U.S. 47, 282.

Martin, R.G. and B.N. Ames. 1961. J. Biol. Chem. 236, 1372.

Marvin, D. and H. Hoffmann-Berling. 1963. Nature 197, 517.

Mitra, S., M.D. Enger and P. Kaesberg. 1963. Proc. Natl. Acad. Sci. 50, 68.

Miura, T. and S. Mizushima. 1968. Biochim. Biophys. Acta. 150, 159.

Nathans, D., G. Notani, J.H. Schwartz and N.D. Zinder. 1962. Proc. Natl. Acad. Sci. U.S.A. 56, 1966.

Nathans, D., M.P. Oeschger, K. Eggen and Y Shimura. 1966. Proc. Natl. Acad. Sci. U.S.A. 56, 1844.

Needham, D.M. and R.K. Pillai. 1937. Biochem. J. 31, 1837.

Nierenberg, M.W. and J.F. Hogg. 1958. Cancer Res. 18, 518.

Nonoyama, M., A. Yuki and Y. Ikeda. 1962. J. Gen. Appl. Microbiol. 9, 299.

Novotny, C., W.S. Knight and C.C. Brinton Jr. 1968. J. Bacteriol. 95, 314.

Ørksov, I. and F. Ørksov. 1960. Acta. Pathol. Microbiol. Scand. 48, 37.

Overby, L.R., G.H. Barlow, R.H. Doi, M. Jacob and S. Spiegelman. 1966b. J. Bacteriol. 92, 739.

Overby, L.R., G.H. Barlow, R.H. Doi, M. Jacob and S. Spiegelman. 1966a. J. Bacteriol. 91, 442.

Paranchych, W. and A.F. Graham. 1962. J. Cell. Comp. Physiol. 60, 199.

Paranchych, W. 1963. Biochem. Biophys. Res. Comm. 11, 28.

Paranchych, W. 1966. Virology 28, 90.

Puck, T.T., A. Garen and J. Cline. 1950. J. Exptl. Med. 92, 65.

Puck. T.T. and B. Sagik. 1953. J. Exptl. Med. 97, 807.

Puck, T.T. and L.J. Tolmach. 1954. Arch. Biochem. Biophys. 51, 229.

Racher, E. 1965. Mechanisms in Bioenergetics. Academic Press, N.Y.

Roberts, R.B., P.H. Abelson, D.B. Cowie, E.T. Bolton and R. B. Britten. 1963. Studies of Biosynthesis in Escherichia Coli, Carnegie Inst. of Wash., Publ. 607, Washington, D.C.

Roberts, J.W. and J.E.A. Steitz. 1967. Proc. Natl. Acad. Sci. U.S.A. 58, 1416.

Sanadi, D.R., D.M. Gibson, P. Ayengar and L. Ouellet. 1954. Biochim. Biophys. Acta. 13, 146.

Scott, D.W. 1965. Virology 26, 85.

Silverman, P.M., H.W. Mobach and R.C. Valentine. 1967. Biochem. Biophys. Res. Comm. 27, 412.

Silverman, P., S. Rosenthal and R. Valentine. 1967. Biochem. Biophys. Res. Comm. 27, 668.

Strehler, B.L. and J.R. Totter. 1952. Arch. Biochem. Biophys. 40, 28.

Trenkner, E., F. Bonhoeffer and A. Gierer. 1967. Biochem. Biophys. Res. Comm. 28, 932.

Valentine, R.C. and M. Strand. 1965. Science 148, 511.

Valentine, R.C. and H. Wedel. 1965. Biochem. Biophys. Res. Comm. 21, 106.

Vasquez, C., N. Granboulan and R.M. Franklin. 1966. J. Bacteriol. 92, 1779.

Watanabe, I. 1964. Nihon Rinsho 22, 243.

Webb, J.L. 1966. Enzymes and Metabolic Inhibitors. Vol. 2, Academic Press, N.Y.

Weber, K. and W. Konigsberg. 1967. J. Biol. Chem. 242, 3563.

Weissmann, C. 1967. The Molecular Biology of Viruses, (Colter and Paranchych, eds.), Academic Press, N.Y.

Wendt, L.W., K.A. Ippen and R. Valentine. 1966. Biochem. Biophys. Res. Comm. 23, 375.

Wick, A.N. and D.R. Drury. 1957. J. Biol. Chem. 224, 963.

Weidel, W. 1958. Ann. Rev. of Microbiol. 12, 27.

Zinder, N. 1963. Perspectives in Virology 3, 58.

B29902